KINETIC AND CLONAL ASPECTS OF IMMUNOLOGICAL MEMORY

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1977
I declare that the experiments described here, and the preparation of this thesis, have been in every respect my own work.
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<tbody>
<tr>
<td>AEF</td>
<td>Allogeneic effect factor</td>
</tr>
<tr>
<td>AFC</td>
<td>Antibody forming cell</td>
</tr>
<tr>
<td>AIS</td>
<td>Anti-lymphocyte serum</td>
</tr>
<tr>
<td>Ars</td>
<td>Arsanilic acid</td>
</tr>
<tr>
<td>ATx</td>
<td>Adult thymectomised</td>
</tr>
<tr>
<td>ATxIBM</td>
<td>Adult thymectomised, irradiated, bone marrow reconstituted</td>
</tr>
<tr>
<td>BA</td>
<td>Brucella abortus</td>
</tr>
<tr>
<td>BkA</td>
<td>Bacterial α-arylase</td>
</tr>
<tr>
<td>BGG</td>
<td>Bovine γ-globulin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund's adjuvant</td>
</tr>
<tr>
<td>CCG</td>
<td>Chicken γ-globulin</td>
</tr>
<tr>
<td>CMI</td>
<td>Cell-mediated immune response</td>
</tr>
<tr>
<td>CML</td>
<td>Cell-mediated lympholysis</td>
</tr>
<tr>
<td>ConA</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CRBC</td>
<td>Chicken red blood cells</td>
</tr>
<tr>
<td>DBS</td>
<td>Dutton's balanced saline</td>
</tr>
<tr>
<td>DH(DTH)</td>
<td>Delayed hypersensitivity</td>
</tr>
<tr>
<td>DIP</td>
<td>3,5 diiodo-4-hydroxyphenacetic acid</td>
</tr>
<tr>
<td>DNFB</td>
<td>2,4 dinitrofluorobenzene</td>
</tr>
<tr>
<td>DNP</td>
<td>2,4 dinitrophenol</td>
</tr>
<tr>
<td>DRBC</td>
<td>Donkey red blood cells</td>
</tr>
<tr>
<td>DT</td>
<td>Dyptheria toxoid</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
</tr>
<tr>
<td>FCA</td>
<td>Freund's complete adjuvant</td>
</tr>
<tr>
<td>FDNFB</td>
<td>1, 5 difluoro 2, 4 dinitrobenzene</td>
</tr>
<tr>
<td>FGG</td>
<td>Fowl γ-globulin</td>
</tr>
<tr>
<td>FUDR</td>
<td>5, fluorodeoxyuridine</td>
</tr>
<tr>
<td>GC</td>
<td>Germinal centre</td>
</tr>
<tr>
<td>GPA</td>
<td>Guinea-pig albumin</td>
</tr>
<tr>
<td>GvH</td>
<td>Graft versus host</td>
</tr>
<tr>
<td>GZ</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>HA</td>
<td>Haemagglutination</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
</tr>
<tr>
<td>HGG</td>
<td>Human γ-globulin</td>
</tr>
<tr>
<td>HBRC</td>
<td>Horse red blood cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
</tr>
<tr>
<td>IFA</td>
<td>Incomplete Freund's adjuvant</td>
</tr>
<tr>
<td>ip</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>125I UdR</td>
<td>125-I-iodo-2-deoxyuridine</td>
</tr>
<tr>
<td>iv</td>
<td>Intravenous</td>
</tr>
<tr>
<td>KLH</td>
<td>Keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>Lac</td>
<td>p-azophenyl-B-D-lactoside</td>
</tr>
<tr>
<td>LPF</td>
<td>Lymphocytosis promoting factor</td>
</tr>
<tr>
<td>LPS</td>
<td>Lypopolysaccharide</td>
</tr>
<tr>
<td>2ME</td>
<td>2-mercaptoethanol</td>
</tr>
<tr>
<td>2MER</td>
<td>2-mercaptoethanol resistant</td>
</tr>
<tr>
<td>MLC</td>
<td>Mixed lymphocyte culture</td>
</tr>
<tr>
<td>MON</td>
<td>Monomeric flagellin</td>
</tr>
<tr>
<td>MSH</td>
<td>Maio squid hemocyanin</td>
</tr>
<tr>
<td>NAD</td>
<td>New antigenic determinants</td>
</tr>
<tr>
<td>NIP</td>
<td>4-hydroxy-5-iodo-3-nitrophenacetic acid</td>
</tr>
<tr>
<td>NNP</td>
<td>3,5-dinitro, 4-hydroxyphenylacetic acid</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PE</td>
<td>Peritoneal exudate</td>
</tr>
<tr>
<td>PFC</td>
<td>Plaque forming cells</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>POL</td>
<td>Polymerised flagellin</td>
</tr>
<tr>
<td>PPD</td>
<td>Purified protein derivative of tuberculin</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cells</td>
</tr>
<tr>
<td>RES</td>
<td>Reticuloendothelial system</td>
</tr>
<tr>
<td>RGG</td>
<td>Rabbit γ-globulin</td>
</tr>
<tr>
<td>SIII</td>
<td>Pneumococcal polysaccharide</td>
</tr>
<tr>
<td>sc</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SRBC</td>
<td>Sheep red blood cells</td>
</tr>
<tr>
<td>sulf</td>
<td>Sulfanilic acid</td>
</tr>
<tr>
<td>TD</td>
<td>Thoracic duct</td>
</tr>
<tr>
<td>TDL</td>
<td>Thoracic duct lymphocytes</td>
</tr>
<tr>
<td>TNP</td>
<td>2, 4, 6 trinitrophenol</td>
</tr>
<tr>
<td>TT</td>
<td>Tetanus toxoid</td>
</tr>
<tr>
<td>1º</td>
<td>Primary</td>
</tr>
<tr>
<td>2º</td>
<td>Secondary</td>
</tr>
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SUMMARY

In a review of the literature, the characteristics of memory cells have been considered in Chapter 1 with particular emphasis on differences between these and virgin cells. Primed and virgin B-cells differ in certain physical properties, cell surface receptors (particularly antigen receptors) and recirculatory behaviour. The relationships between virgin and memory cells, the kinetics of priming and duration of memory were also considered with respect to RBC and protein antigens and their persistence in the body.

In Chapter 2, an anti-DNP PFC assay was established using dinitrophenylated-SRBC, covalently coupled with low concentrations of dinitrofluorobenzene (DNFB).

When coupled with high concentrations of DNFB, both CRBC and SRBC proved excellent for immunising mice subcutaneously against DNP and both primary and secondary responses were compared with those to DNP-protein conjugates. Primary IgG anti-DNP PFC developed a little earlier in response to immunisation with DNP-CRBC than to DNP-BGG.

Certain aspects of the carrier effect were also investigated. Challenge with a different DNP-conjugate to that used for priming usually resulted in a slightly delayed secondary response. This delay could be circumvented by additional immunisation with the second carrier at the time of priming with the DNP-primary carrier conjugate, presumably by provision of primed T-cells. However, carrier priming alone resulted in suppression of the primary anti-DNP IgG response to DNP-CRBC.

Controversies concerning the development of memory to RBC antigens were outlined in the Introduction to Chapter 3 and
experiments were designed in an attempt to reconcile these differences. At varying intervals after subcutaneous priming with SRBC, draining lymph node cells were adoptively transferred to X-irradiated recipients for assay of their capacity to give a secondary response. Memory to SRBC reached a maximum, in terms of both number of primed cells and clonal heterogeneity of the memory cell pool, within 1-2 weeks of priming and remained reasonably constant over the next 4-6 weeks.

These experiments were extended to include a comparison of the kinetics of priming to DNP when conjugated to RBC (CRBC) or protein (BGG) carriers. It was hoped that this approach might elucidate factors which affect the kinetics of priming. No anti-DNP memory was detected until the third week after priming with DNP-CRBC but memory was evident within one week of priming to DNP-BGG.

In Chapter 4, the phenomenon of immunological pre-emption was considered with respect to its dependence on suppressor or helper T-cells. An attempt was made to prime the cells responsible for immunological pre-emption and compare the kinetics of priming with those for T-helper cells. The degree of suppression of the test subcutaneous antigen injection was presumably related to the number of helper and/or suppressor cells activated by the intraperitoneal antigen, and proved extremely dose-dependent. For this reason, priming for pre-emption was not easily demonstrated but could be detected within 2-7 days of priming. Thereafter there was no further increase. This correlates with helper T-cell priming but leaves open the question of suppressor priming.

In Chapter 5, further information was provided on the action
of the adjuvants \textit{C. parvum} and \textit{B. pertussis}, by studying their effect on clonal size and heterogeneity of the anti-SRBC primary response and the memory cell pool. Initially it was confirmed that \textit{C. parvum} exerts its greatest adjuvant effects when injected intraperitoneally (ip) at the same time as ip antigen. In addition, the subcutaneous route was investigated and \textit{C. parvum} was shown to be most effective when injected subcutaneously (sc) 4 days prior to sc antigen. \textit{B. pertussis} was injected at the same time as antigen via both ip and sc routes. Both adjuvants exerted their enhancing effects primarily by prolonging the primary response, perhaps by suppressing or circumventing control mechanisms. Individual clones were well-expanded and there was no evidence for recruitment of new clones.

At the same time the memory cell pool was expanded, as shown by \textit{in situ} challenge or adoptive transfer 5 weeks after priming. \textit{C. parvum} may still have abrogated control mechanisms at that time but there was no such indication for \textit{B. pertussis}.

When injected ip prior to antigen, \textit{C. parvum} suppressed the primary response to SRBC ip but had no effect on the size and clonal nature of the memory cell pool.

In Chapter 6, certain recirculatory properties of memory cells were considered and related to the presence of persistent antigen. Mice were primed in the front footpads and the memory capacity of the draining (brachial and axillary) and non-draining (inguinal and popliteal) nodes were compared by their adoptive transfer to X-irradiated recipients. It was confirmed that memory to SRBC was fully developed in the draining nodes within the first week of priming and that the memory pool encompassed a broad spectrum of clones. No secondary response was seen after transfer of recently
primed (7 days) cells from non-draining nodes but during the following 2 weeks the capacity to give a secondary response developed, presumably by recirculation of memory cells from the draining lymph nodes. There was some evidence that this was an antigen dose dependent phenomenon, memory in the non-draining nodes equalling that in the draining nodes (both in terms of number of primed cells and clonal heterogeneity) within 14-21 days, depending on the priming SRBC dose.

A hapten-carrier system was devised to compare the recirculation of memory T and B-cells in the same animal. 5 weeks after priming, T-cells freely recirculated and were available to help in a secondary response in both draining and contralateral nodes. However B-cells primed at the same time, gave a more rapid response in the draining than non-draining nodes indicating that there may be a "sessile" population of memory cells, dependent on persistent antigen, which went undetected by the adoptive transfer technique.
Immunological memory is a definitive property of the immune response (Miller 1973). The phenomenon has been recognised and utilised for protection against disease for many years, dating back to the practice of variolation in ancient India and China; a practice which involved inoculation of live smallpox organisms from disease pustules and often proved extremely dangerous. It was the 18th century before a safe vaccine for smallpox was developed by Jenner (1798) following his discovery that immunisation with the non-virulent cowpox afforded protection against subsequent smallpox infection. Nearly a century later Pasteur (1887) developed a vaccine for anthrax using attenuated organisms and thereby set the scene for modern vaccination procedures, most of which use a harmless form of the antigen. The late 1800's also brought the discovery of antibodies and phagocytes, and the observation that phagocytosis was greatly enhanced by immunisation (Denys & Leclef 1895). However it was not until the importance of lymphocytes in immunity became established, and more sophisticated techniques developed for measurement of immune responses, that scientists could begin to unravel the cellular basis of immunological memory. Information has accumulated rapidly in recent years and this first chapter aims to survey the current status of the field with particular reference to aspects covered in this thesis.

Memory has been demonstrated for allograft rejection (Sprent & Miller 1976a and b), cytotoxicity (Andersson & Häyry 1974; Häyry & Anderson 1974; Koren, Wunderlich & Inmar 1976; Wagner & Rollinghoff 1976) and delayed hypersensitivity (DH) reactions (Benacerraf & Levine 1962; Gell & Silverstein 1962) as well as for the humoral antibody response (Miller & Sprent 1971).
This study is concerned solely with humoral immunity, so is limited to considerations of memory carried by B-cells, T-helper cells and T-suppressor cells.

A secondary antibody response, reflecting the presence of memory, can be distinguished from a primary response to both soluble and particulate antigens by:

1) a reduced or absent lag phase before appearance of circulating antibodies and antibody forming cells (AFC);
2) higher peak numbers of AFC and titres of circulating antibody;
3) a bias in favour of IgG antibody (for T-dependent antigens).

(review: Celada 1971)

Co-operation between T and B-cells is essential for a secondary antibody response to T-dependent antigens (Miller & Sprent 1971; Mitchell et al. 1972). Memory has been demonstrated in both T and B-cell lines (reviewed: Miller 1973) and primed T and B-cells not only co-operate with one another but also with virgin cells of the other class under appropriate conditions (reviews: Katz & Benacerraf 1972; Bullock & Möller 1974).

There is some controversy over the actual effect of primed T-cells on the B-cell response. Some workers believe that T-cells merely augment the response, while others suggest a controlling role for these cells over class and affinity of antibody produced. This will be considered in more detail later.

It is clear that one important feature of the development of both T and B-cell memory is the expansion of antigen-sensitive cell pools. However a qualitative change may also occur in primed cells since they respond more rapidly than virgin cells and the class and affinity of secondary antibody differs from that in the primary response (Miller & Sprent 1971; Marrack &
Kappler 1976). In addition memory involves changes in parameters such as antigen handling and localisation, cell recruitment and retention by antigen. These will be considered in later sections.

**Characteristics of memory cells**

Memory is carried by small lymphocytes. This was first shown for the secondary antibody response by Gowans and Uhr (1966), who removed the large cells from primed rat thoracic duct lymphocytes (TDL) and transferred the remaining small lymphocytes into X-irradiated recipients for challenge. They gave an excellent secondary response. More recently, Strober (1976) has shown that B 'blast' cells in the TDL are also memory cells. These blast cells are 4-6 times more efficient at antibody production than are small memory lymphocytes when compared on a cell per cell basis in adoptive transfer. However, since they constitute only 7-8% of the TDL their contribution to the memory response of unfractionated TDL is only small.

The memory B-cell does not seem to be derived from an antibody forming cell. Preparations of AFC purified either on cotton wool columns or by micromanipulation from the centre of hemolytic plaques did not transfer memory (Celada 1971). Loss of adhesion to cotton wool is one of the ways in which B-memory cells can be distinguished, and separated, from unprimed cells (Schrader 1974; L'Age-Stehr et al 1976; Schrader & Vadas 1976). Memory B-cells also differ from unprimed cells in buoyant density and electrophoretic mobility (L'Age-Stehr & Herzenberg 1970; Schlegel et al 1975). It is not yet certain whether T-memory cells also show these particular properties. Subsets of T-cells have been separated on buoyant density gradients but probably do not relate to virgin and memory cells (Schlesinger et al 1975). Other
T-cell subsets with fast and slow electrophoretic mobility have been described, the slow ones becoming predominant in later life, thus possibly representing T-memory cells (Droege 1976).

Perhaps the two most emphasised differences between virgin and memory B-cells are the increase in lifespan of the memory cell and also the capacity of memory cells to recirculate from blood to lymph. It is, as yet, uncertain whether T-virgin and T-memory cells can be separated on the same basis. Raff & Cantor (1971) subdivided T-cells into two populations, T1 and T2 cells. The former are short-lived after adult thymectomy (ATx), insensitive to anti-lymphocyte serum (ALS) and found predominantly in the thymus and spleen. The latter are long-lived, ALS sensitive and recirculate from blood to lymph. They originally suggested that T1 cells may be virgin cells, and are converted to memory T2 cells upon contact with antigen, a proposition which is strongly supported by Araneo et al (1976). However others have shown that the long-lived (T2) cell is responsible for helper activity but that the short-lived (T1) cell is required for priming (Kappler et al 1974). It is clear that at the moment these properties cannot be used with any confidence to separate unprimed and primed T-cells.

Virgin B-cells have a generation time of less than 48 hours but that of memory B-cells is considerably longer (Strober 1972). Suicide of dividing cells by 3H-thymidine treatment of donor rats for 48 hours reduced the adoptive primary response by more than 100 fold, while the secondary response was reduced only 2 fold. Memory B 'blasts' have a similar lifespan to virgin cells (Strober 1976). Administration of 3H-thymidine over long periods of time has indicated that B-memory cells have a lifespan of about
8 weeks - less than that of T-memory cells which has been estimated at 16 weeks (Sprent & Miller 1972). Some virgin cells, found among memory B-cells, are present in the TDL, but they do not recirculate from blood to lymph (review: Strober 1975). This was shown by passage of TDL from either virgin or 2, 4 dinitrophenol (DNP)-primed animals through intermediate recipients. TDL from these recipients were collected and transferred to a final carrier-primed host for assay. Virgin cells gave an extremely poor response when compared with the unpassaged controls whilst memory cells gave a much enhanced response.

At this point a word of warning is required. Clear-cut differences are seldom seen between virgin and memory cells when comparing electrophoretic mobilities, adherence properties, buoyant densities, recirculation or lifespan. Unprimed animals often contain some cells with slow mobility, low adherence, long-lifespan or ability to recirculate. These exceptions have been attributed to unintentional environmental priming. The choice of antigen is therefore important. Strober (1975) showed that whilst virgin B-cells with ferritin specificity were truly short-lived and non-recirculatory, those for sheep erythrocytes (SRBC) were both long-lived and recirculatory, and those for DNP were intermediate in that they were long-lived but non-recirculatory. However, recent evidence from thoracic duct drainage of thymectomised foetal lambs indicates that some B-cells recirculate in the absence of any possible environmental priming (Pearson et al 1976).

Antigen receptors and triggering of memory cells

The identity of the memory B-cell receptor for antigen has provoked much research and considerable controversy in recent
years. The evidence cited above suggests that many, if not all, recirculatory B-cells form part of the memory cell pool. These represent the majority of thoracic duct B-cells, thus a study of B-cells among the TDL should provide information on the memory cell receptor. Estimates of the proportion of B-cells among the TDL range from 10-30% (Sprent 1973; Parish & Hayward 1974). More than 80% of these carry IgM receptors (Mason 1976), and this sub-population includes IgG memory cells. This was shown by Abney et al (1976) who found that in vivo treatment with anti-μ eliminated almost all IgG memory while anti-IgGl, 2a, 2b and D had little effect. In similar experiments, Pierce et al (1972a and b) showed that anti-μ eliminated early memory cells but by the tenth day after priming IgG memory was resistant to this treatment. However over the next few weeks anti-μ sensitivity again developed.

Separation of IgG+ cells from TDL using a fluorescence activated cell sorter (FACS) has given contradictory results to those of Abney et al in that 90% of the IgG memory was found to be carried by the 3% of IgG+ bearing TDL (Mason et al 1976). Strober (1976) has recently shown that memory B 'blasts' carry IgM surface receptors whilst memory small lymphocytes carry IgG receptors but this observation does not explain the function of the majority of IgM+ TDL.

Antibody avidity increases with time after primary immunisation although some low avidity antibody is detected throughout the response (Eisen & Siskind 1964; Kim & Siskind 1974). This preferential stimulation of high avidity cells has been explained by increasing competition between cells for waning amounts of antigen. In a secondary response, the avidity
of anti-DNP antibodies is high within a few days of boosting (Gelada 1971). This could be the result of maturation of the memory cell pool in parallel with the primary response so that late after priming the memory population consists predominantly of high avidity cells. There is some evidence for this in that extremely low doses of antigen can stimulate late memory cells - more than a 10 fold difference has been found in the HSA (human serum albumin) concentration required to stimulate 6 week and 16 week memory cells (Valantova et al 1967). This maturation is probably antigen dependent since transfer of 2 week primed cells to X-irradiated recipients showed no difference in antibody avidity whether challenge was immediate or delayed for 6 weeks (Feldbush & Gowans 1971).

The gradual selection for memory cells capable of producing high avidity antibody does not involve elimination of low avidity memory cells. However, the presence of pre-formed antibodies at the time of antigen challenge may reduce the booster injection to such a small effective dose that only high avidity cells are triggered. The presence of low avidity cells was shown by adoptive transfer experiments where low avidity antibody was induced, provided the antigen dose was sufficient large (Macario et al 1973; Feldbush & van der Hoven 1976).

The number of receptors on B-memory cells appears to be higher than on cells stimulated in a primary response. The evidence for this comes from studies on antigen-dose requirements for triggering DNP-memory cells (Klinman 1972), requirement for primed T-cell help (Klinman 1972), tolerance induction by monovalent hapten and receptor blockade by DNP-SIII (Klaus & Humphrey 1975; Klaus 1975). Klaus & Humphrey (1975) showed
that binding of monovalent DNP to memory cells differed very little from the spectrum of binding to virgin cells, but that the cellular avidity for binding DNP-SIII was very much higher.

Evidence has recently been presented which strongly implicates the helper T-cell in selection of memory cells capable of producing high avidity antibody (Okumura et al 1976). In the absence of helper T-cells, memory cells were generated, but 2° antibody produced by these cells was of low avidity. It is generally agreed that T-cell help is required for the expression of B-cell memory (Safford & Tokuda 1971; Schlegel 1974), and there is some evidence that carrier-primed T-cells can bias an anti-hapten response towards IgG production and high avidity antibodies (Miller et al 1971; Cunningham & Sercarz 1971; Cheers & Miller 1972; Kishimoto & Ishizaka 1973; Hurme et al 1973). However others have shown that the priming schedule for the hapten governs the class distribution and avidity of secondary antibody while carrier priming serves only to amplify this response (Schirrmacher & Rajewsky 1970; Cudkowicz et al 1969; Shearer et al 1969). Klinman (1972) has suggested a compromise solution, namely, that primed T-cells serve mainly to amplify the response of monofocal antibodies, but that they also prevent tolerance induction of high avidity clones thereby biasing the response in favour of high avidity antibody. However the late secondary response is less primed T-cell dependent than the early response (Sprent & Miller 1971; Schlegel 1974; Bullock & Möller 1974).

**Priming and duration of memory**

It is still uncertain in which part of the lymph nodes and spleen memory cells are generated. The germinal centre (GC) was originally implicated since antigen is retained on the plasma
membrane of the GC reticular cells (Hanna & Szakal 1968; Sordat et al 1970), antigen-antibody complexes localise in the germinal centres (Ada & Williams 1966; Herd & Ada 1969b), and specifically primed cells are effectively localised by retained antigen (Ponzio et al 1975; Emeson & Thursh 1971 & 1972). Histological damage to germinal centres by chemicals or X-rays correlates with a decrease in memory (Celada 1971). Prevention of GC growth by specific antibody given 24 hours after antigen also prevents 7S memory development (Hanna et al 1969). However this is only circumstantial evidence for the importance of GCs in memory development. It now seems more likely that memory cells are generated elsewhere and that the GC acts as a site for memory cells to "home" to, either in response to retained antigen or following a secondary challenge with antigen (Pelo et al 1972).

The X-Y-Z scheme of immune cell maturation was originally proposed to account for the relationship between unprimed lymphocytes, memory cells and antibody forming cells (Sercarz & Byers 1967). The X-cell represents a virgin cell which, upon contact with antigen, undergoes blast transformation to become either an APC or a memory cell depending upon environmental conditions. They based this model on their observations that memory does not develop to the same extent after an antigen dose which gives a high primary response. More recent data also suggest that the early Y-cell compartment is inversely related to the antigen dose used for immunisation. A high dose of SRBC (5 x 10^8 ip) has been shown to deplete early IgM memory cells presumably by incorporation of these cells into the primary response (Black & Inchley 1974). This effect was not found after the lower dose of 5 x 10^5 SRBC.
No scheme for T-cell memory development has been proposed. It is indeed possible that T-cells follow a similar X-Y-Z scheme with Z cells being effector T-cells or helper T-cells. *In vitro* studies of blast transformation in mixed lymphocyte culture (MLC), cell-mediated lympholysis (CML) and phytohaemagglutinin (PHA) responsiveness have all shown that T-blasts revert to small lymphocytes with memory characteristics (Andersson & Häyry 1974; Häyry & Andersson 1974; Macdonald *et al* 1974; Macdonald *et al* 1975). *In vitro* studies of LPS stimulation of B-cells have recently shown that under certain conditions most of the stimulated B-cells will revert to small lymphocytes after blast transformation whilst only a few become AFC (Askonas *et al* 1976b). The memory characteristics of these small lymphocytes have not yet been tested. It may well be that the T-cell *in vitro* systems are analogous to this, and that blast transformation is yielding a few effector cells and many memory cells.

The rate of memory cell development to SRBC has been studied by Black & Inchley (1974). They showed that IgM memory to SRBC develops within the first 24 hours of priming without cell division, and is maintained for 4-5 days before gradually declining to primary levels. Others (Cunningham & Sercarz 1971) have shown a late rise in IgM and IgG memory which continues for several weeks after priming. This will be considered further in Chapter 3. IgG memory to SRBC is detected within 4 days of priming and usually peaks on the 5th day (Black & Inchley 1974). B-cell division is required on days 0, 1, 2 and 3 but T-cell division is only required up to day 2. These experiments involved blockade of the primary response at different times.
after antigen injection, using passive antibody and cyclophosphamide. Mice were challenged 12 weeks later to assess the degree of memory development between the time of priming and suppression. The results for early generation of both IgM and IgG memory are in general agreement with those of others using different systems to study the rate of memory generation to SRBC (Cunningham 1969a and b; Cunningham & Sercarz 1971; Mond et al 1974; Romano et al 1975).

Memory generation to proteins follows a similar X-Y-Z scheme but is not usually detected until a little later (day 7-10 after priming) and often continues to rise for several weeks, or even months (Vischer & Stastny 1967; Hamaoka et al 1969; Jacobson & Thorbecke 1969; Cerottini & Trnka 1970; Stavitsky & Folds 1972). The kinetics of priming to RBC and protein antigens are considered in more detail in Chapter 3.

The possibility that T-cell help is required for memory B-cell generation is raised by the finding that T-cells are fully primed by day 3 after antigen, and B-cells not for at least another day. Support for this comes from studies showing that anti-carrier antibody not only prevents T-B cooperation for a primary response but also prevents B-cell memory generation (Hamaoka et al 1973; Takatsu et al 1974). Memory generation has been studied in nude mice (Roelants & Askonas 1972; Diamenstein & Blitstein-Willinger 1974; Schrader 1975), adult thymectomised, bone marrow reconstituted (ATxBM) mice (Schrader 1975), allotype suppressed mice (Okumura et al 1976) and also in normal mice to both T-independent antigens (Mond et al 1974) and to hapten-carrier conjugates with high epitope densities (Vachek & Kolsh 1975). These experiments have
shown that normal B-memory cell pools develop, but memory cannot
be expressed, in the absence of T-helper cells. There is one
exception to these in that Schlegel (1974) found that T-cells
were required for IgG memory development to NIP in nude mice.

Memory to certain antigens can still be detected many months,
or even years, after priming. This could be due to
a) extremely long-lived memory cells
b) slow turnover of memory cells to retain a constant pool size
c) recruitment of new memory cells by persistent antigen.

Sprent and Basten (1973) have shown that the lifespan of memory
B-cells in the mouse is approximately 6-8 weeks. The fact that
memory persists for several months implies that there must be some
turnover of memory cells, perhaps in response to persistent
antigen. To avoid any influence of persistent antigen, memory
cells have been transferred to X-irradiated recipients for various
times before challenge. Original experiments indicated that
there were two populations of memory cells, one short-lived and
the other long-lived (Celada 1967; Feldbush & Gowans 1971;
Feldbush 1973). More recently Feldbush et al. (1974) have shown
that the short-lived population is an artefact of the cell transfer
system used. They concluded that there is one population of
B-memory cells with a functional half-life of approximately
40 days in the absence of antigen (Feldbush 1973). Elson, Jablonska
and Taylor (1976) have developed a double transfer system in which
they culture memory cells in a normal, unimmunised host for various
periods before transfer to an X-irradiated host for challenge.
They have used this system to compare the functional half-lives
of virgin and memory cells by transferring virgin, or 2-3 month
trinitrophenol (TNP)-primed B-cells from CBA-Igl^{bb} donors into CBA-Igl^{aa} recipients. After various intervals of time, cells from these intermediate recipients were adoptively transferred to irradiated hosts for challenge. Results from these experiments indicated that virgin cells have a half-life of less than 7 days, and memory cells 14-21 days. If virgin cells have a turnover rate of 24-48 hours (Strober 1972) they must have a limited capacity for self-renewal in the absence of antigen. However Strober found that DNP "virgin" cells actually had a slow turnover rate and this may also be true for cells specific for TNP.

When memory is detected for several months after priming it must either involve gradual turnover and renewal of memory cells, or must depend on recruitment of new memory cells. There is evidence for antigen persisting in the lymph nodes and spleen for many months, and for recycling of memory cells to maintain or increase the memory pool following antigen boost. Induction of spontaneous antibody synthesis in lymph node cultures many months after priming has often been observed and is probably related to antigens still present in those nodes, this antigen stimulating memory cells to respond, once released from their *in vivo* control (Stecher & Thorbecke 1967; Tew *et al* 1973; Stavisky *et al* 1974; Greene, Tew & Stavitsky 1975). Another indication that antigen persists for long spells is the continual rise of memory to some antigens for several weeks after priming (Jacobson & Thorbecke 1969; Cunningham & Sercarz 1971; Stavitsky & Folds 1972). In addition, secondary responses remain higher in lymph nodes draining the site of an antigen injection than in non-draining nodes for many weeks after priming (Jacobson & Thorbecke
1969; Stavitsky & Folds 1972) strongly suggesting a role for antigen in the maintenance of memory. This will be considered in more detail in Chapter 6.

Feldbush and van der Hoven (1976) have shown that following in vivo transfer of primed cells to X-irradiated recipients, a low dose of antigen given at the time of transfer can potentiate the secondary response and prevent the decline seen in the absence of antigen. This was less pronounced after a higher antigen dose presumably because more memory cells were driven to AFC and less recycled to form new memory cells. Several groups have propagated particular anti-DNP clones through 5-6 transfer generations and have shown that memory B-cells generate new memory B-cells following the same X-Y-Z scheme as for virgin cells (Askonas & Williamson 1972; McMichael & Williamson 1974; McMichael & Willcox 1975).

Following continual antigen boosts Cramer and Braun (1975) have shown that existing memory clones to streptococcal group polysaccharides are amplified rather than new clones recruited. However there is evidence that new memory clones to NIP are recruited for at least 72 days after priming despite the fact that the memory pool attained a maximum size within 30 days (Kreth & Williamson 1973). There is thus some evidence both for recycling of memory cells and new cell recruitment in response to persisting antigen. Strober (1976) has recently shown that memory B 'blasts' can only be detected in the TDL for a few weeks after antigen injection whilst small memory cells can be detected for several months. He suggested that these blasts may be continually generated in response to persistent antigen,
but that long-term small memory lymphocytes are less dependent on this antigen. The significance of these observations will remain uncertain until the relationship between memory 'blasts' and small lymphocytes has been established.

**Aim of study**

The experiments described in this thesis were designed to investigate certain outstanding questions of memory development and deployment. Although well-tried techniques of adoptive transfer and AFC assay have proved useful, emphasis has been given, in particular, to evaluation of B-cell memory in clonal terms since this approach has not been greatly used in other work. It is applicable to problems such as the late recruitment of memory cells to certain antigens, and the relative influence of adjuvants on the proliferation of B-memory and antibody forming cells. It has been of interest to use the same approach to study the distribution of memory cells, since concentrations of a few, high affinity clones in draining lymphoid tissue would tend to support the role of persistent antigen in controlling memory generation.

A further area of controversy to which attention has already been drawn, is the rate of memory generation to different antigens. Now that suitable techniques are available, it seemed of great interest to compare the generation of B-memory to a single hapten on different carriers, as well as to the carrier determinants themselves.

Although most of the project concerns B-cells, some studies on T-cell priming have also been conducted, with an emphasis on the priming of suppressor populations. The areas chosen for study thus include:
1. The development of clonal memory to SRBC and a comparative study of the kinetics of priming to RBC and protein antigens. (Chapter 3)

2. Priming for suppressor T-cells. (Chapter 4)

3. The effect of adjuvants on the clonal response to SRBC and on priming to SRBC. (Chapter 5)

4. Studies on sessile and recirculatory memory cells. (Chapter 6)

These topics will be considered in more detail in the individual chapters.
CHAPTER 2

IMMUNISATION AND ASSAY WITH DNP-RBC CONJUGATES
INTRODUCTION

In contrast to responses to natural antigens with multiple determinants, responses to chemically defined haptens, coupled to an appropriate carrier (Landsteiner & Simms 1923), are less heterogeneous and more easily understood. For this reason they have greatly increased our understanding of the mechanisms of antibody production. A large amount of attention has been given to variables such as epitope density and its influence on the induction of immune responses. In the context of immunological memory, hapten-carrier systems have been chosen in this thesis to study two aspects of the behaviour of primed lymphocytes. These are:

a) to define the relative importance of epitope and carrier in governing the kinetics of B-cell priming, by comparing the responses to DNP (dinitrophenol) when conjugated to either a protein or red blood cell (RBC) carrier (Chapter 3), and

b) to study the recirculatory properties of T and B memory cells (Chapter 6).

Certain aspects of the immune response to hapten-carrier conjugates have been adequately reviewed by Bullock and Möller (1974). In particular they have considered the nature of the cells responding to both the hapten and the carrier, with emphasis on interactions between these cells and control of anti-hapten responses by carrier-specific cells. Some of these points will be reconsidered and updated in this chapter.

T and B-cell responses to hapten-carrier complexes

It is now well established that in a response to a T-dependent hapten-carrier conjugate, B-cells make antibodies to
both the hapten and carrier whilst T-cells responsive to the carrier co-operate with both anti-carrier and anti-hapten B-cells (Mitchison 1971b; Bullock & Müller 1974). B-cells specific for the hapten alone have been demonstrated both by the production of hapten-specific antibody and by the binding of hapten-nonimmunogenic or T-independent carrier complexes to the B-cell receptor (Hamilton & Miller 1973; Katz et al 1974). Such binding leads to tolerance of hapten-specific B-cells (Klaus & Humphrey 1974). It has been suggested that the normal role for carrier-specific 'helper' T-cells is to provide a signal which prevents tolerance induction by cell bound antigen (Hamilton & Miller 1973; Mitchell 1974; Bullock & Möller 1974).

That T-cells responsive to the carrier were important for anti-hapten responses was first shown in experiments involving reconstitution of X-irradiated mice with either immune spleen cells or anti-Ø plus complement treated spleen cells. The anti-hapten response was much reduced by removal of primed T-cells in this way but could be restored to normal secondary ($2^{0}$) levels by addition of carrier-primed spleen cells from another donor (Raff 1970). Carrier-specific educated T-cells can substitute for primed spleen cells (Cheers & Miller 1972) and, under certain circumstances, so can T-cell factors produced during a Graft versus Host (GvH) response (allogeneic effect factor, AEF) (Armerding & Katz 1974). Hapten-specific T-cells have been reported, and can provide a low level of help for an anti-carrier response by carrier-primed, T-depleted spleen cells (Doughty & Klinman 1973). It is possible that such a small determinant activates only low numbers of helper T-cells and that their effects are normally totally masked by carrier-specific T-cells (Bullock & Möller 1974).
The "carrier effect"

The "carrier effect" is the term used to describe the dependence of a 20 anti-hapten response on priming with the same carrier as that used for challenge. It was originally described in connection with guinea-pig delayed hypersensitivity (DH) responses to hapten-carrier conjugates and was not thought to apply for the antibody response (Benacerraf & Levine 1962). A comparison was made of the DH and Arthus (antibody dependent) reactions of TNP-GPA (trinitrophenylated guinea-pig albumin) primed guinea-pigs to a challenge with either TNP-GPA or TNP-OVA (ovalbumin). Challenge with the homologous conjugate (TNP-GPA) elicited good DH and Arthus reactions whilst challenge with the heterologous conjugate (TNP-OVA) only induced an Arthus reaction, slightly reduced in intensity. Insertion of an e-amino capryl chain between hapten and carrier also totally abolished the DH response (Benacerraf & Levine 1962) but has little effect on the antibody response (Mitchison 1971b). It would seem that the attachment area of the hapten to GPA is extremely important and that a DH response may involve recognition of a hapten plus some of its surrounding carrier.

There is now evidence for a carrier effect in the antibody response also, but this is most pronounced when challenge takes place soon after priming (Bullock & Möller 1974) or when cells are transferred to X-irradiated recipients for challenge (Mitchison 1971a). The gradual reduction in carrier effect with time after priming probably explains the inability of early workers to demonstrate it. Several workers have demonstrated the importance of the attachment region of the hapten to the carrier molecule for the antibody response (Henney 1970; Strausbach et
al 1972; Rubin 1973). Antibodies are formed to new determinants in the region of attachment (NAD), and are probably normally detected as low affinity antibody to the hapten. Henney (1970) showed that antibodies directed towards NAD predominated in the early 1° response of guinea-pigs to DNP$_{12}$ HGG. Anti-DNP antibodies were not detected until 2 weeks after immunisation but by 5-6 weeks these anti-hapten antibodies predominated, the proportions at that time being approximately 50% anti-DNP, 40% anti-NAD and only 10% anti-carrier. The progression towards predominantly anti-hapten antibody probably parallels that of affinity maturation of the response with time after priming (Valantova et al 1967). Assuming that a similar maturation might occur within the memory cell pool, a predominance of NAD-specific memory cells soon after priming and of hapten-specific memory cells late after priming, could explain the gradual reduction in carrier effect with time after priming.

More recently it has been shown that while T-B co-operation is essential for a 2° response, with time after priming there is a gradual diminution in the dependence of B-memory cells on help from primed T-cells. For instance, Schlegel (1974) found that when NIP-HGG primed cells were transferred to X-irradiated recipients at different intervals after priming, and the recipients challenged with NIP-POL (polymerised flagellin), a low 2° response was detected with 5-6 week primed cells, but a high response with 10 week primed cells. This 2° response was dependent upon help from unprimed T-cells, but the response of 5 week primed cells could be boosted 10-15 fold by prior priming of the recipients with monomeric flagellin (MON) to provide memory T-cells. The response of late (10 week) memory cells, on the other hand, was not much
affected by MON priming. It is possible that high avidity memory B-cells can be stimulated with the help of unprimed T-cells whilst low avidity cells require help from primed T-cells.

The fact that a higher 2° response is usually found after challenge with the homologous conjugate, particularly after adoptive transfer, is probably related to the greater degree of help provided by primed T-cells. Antigens differ in their ability to act as good heterologous carriers, possibly due to differing degrees of primary (1°) T-cell stimulation (Mitchison 1971a) and therefore in the degree of help provided by 1° T-cells. High challenge doses of heterologous conjugate often overcome the carrier effect presumably by more efficient stimulation of 1° T-cell help.

In addition, Bullock and Möller (1974) have suggested that the late decline in carrier dependence might be due to a gradual emergence of hapten-specific T-cells which may have been tolerised early in the immune response, but may obviate the need for carrier-specific helper cells later on. They also put forward the idea that eventual unmasking of persistent antigen might restimulate memory T-cells. There is some evidence for this from observations of Strausbach et al (1972) that carrier challenge alone could restimulate anti-hapten antibody production. However these authors prefer to explain this as stimulation of anti-NAD antibodies by the carrier.

Regulation of antibody class and affinity in secondary responses

The influence on B-cells of primed T-cells has been held to include control of factors such as antibody class and affinity & Paul (Gershon/1971; Cheers & Miller 1972; Hurme et al 1973) and the affinity maturation of memory cells (Kishimoto & Ishizaka 1973; Okumura et al 1975) as well as overall antibody output (Klaus &
Cross 1974). However T-cell regulation of B-cell responses remains a controversial topic, and their direct influence may be limited to only some of these factors.

For instance, it has been suggested that the predominantly IgM response to T-independent antigens, or to T-dependent antigens in nu/nu mice, reflects the importance of T-cells for IgG production. However Schirrmacher and Rajewsky (1970) have shown that the balance between $2^9$ 7S and $19^8$ anti-sulf antibody, after challenge with sulf-HGG, depended not on carrier priming, but on the conditions of B-cell priming with sulf-BSA. Others have reached the same conclusion, namely that in a hapten-carrier system, IgG production is not appreciably influenced by priming with free heterologous carrier (Katz et al 1970; Kishimoto & Ishizaka 1972). The theory that T-cells can directly influence antibody class is supported by the finding that a non-specific signal, generated in a GvH reaction, could induce IgG production to DNP-SIII (Klaus & McMichael 1974).

However, since IgG production to SIII alone could not be initiated, these experiments suggest that while T-cells may influence its expression, it is the B lymphocyte itself which determines antibody class.

One way in which primed T-cells might influence antibody class or affinity indirectly follows from their ability to augment antibody output, for which there is good evidence from hapten-carrier systems (Kishimoto & Ishizaka 1972; Falkoff & Kettman 1972; Hurme et al 1973; Klaus & Cross 1974). Increased antibody production would be expected to result in earlier selective pressures exerted on the cell population by factors such as antibody feedback. According to the scheme of Siskind et al (1968) a faster selection of high affinity cells would follow,
giving an apparent bias in favour of high affinity IgG. This would be in accord with the observation of Hurme et al (1973) that augmentation of the anti-NIP-OVA response by OA priming also favours high affinity IgG production. If modification by antibody feedback control is involved, then it depends on anti-hapten rather than anti-carrier antibodies, since the latter tend to reduce the amount and affinity of new anti-hapten IgG (Hurme et al 1973).

The importance of epitope density in determining the nature of the anti-hapten response

When the epitope density of a conjugate is high much of the carrier molecule will be masked, with the result that the class and affinity of anti-hapten antibody produced to a high density conjugate may differ from that produced to a low density conjugate. It has been suggested that at high epitope densities carrier recognition might be abolished and the hapten-carrier conjugate may act as a T-independent antigen.

A typical T-independent response, consisting predominantly of IgM with little or no IgG, is obtained when DNP is conjugated to the T-independent antigens SIII, levan or Ficoll (Klaus & Humphrey 1974; Klaus et al 1976). This is also typical of the responses to high epitope density conjugates of haptens with T-dependent antigens, for example NIP\textsubscript{34}BSA and DNP\textsubscript{50}BSA (Aird 1971; Klaus & Cross 1974). In contrast, the low density conjugates NIP\textsubscript{2}BSA and DNP\textsubscript{5}BSA induce predominantly IgG responses whilst DNP\textsubscript{31}BSA induces a transitional type of response in which IgG predominates only after a high immunising dose (Klaus & Cross 1974). Sarvas & Makela (1974) have shown that polyvalent conjugates are essential for IgM production but IgG can be induced by both monovalent (NIP\textsubscript{<1.5}OA, NIP\textsubscript{<1.4}BSA) and polyvalent conjugates.
The same has been shown for the TNP-SRBC response, heavily conjugated erythrocytes inducing IgM and IgG but lightly conjugated erythrocytes inducing IgG only (Naor et al. 1974). However other results with haptened erythrocytes are less clear, probably because the efficiency of conjugation varies with methods used and it is hard to assess the exact epitope density. Kettman and Dutton (1970) could only get a response by immunisation with heavily coupled RBC and Makela et al. (1970) found that low coupling favoured IgM. Though there are exceptions, it is probably fair to make the generalisation that low epitope densities favour IgG production whilst high epitope densities favour IgM.

Further support for the T-independent nature of the responses to high density conjugates comes from the usually poor antibody response to the carrier molecule or to the NAD (Rubin et al. 1973; Klaus & Cross 1974) which indicates that the carrier molecule is inaccessible to B-cells. Despite this apparent T-independence, studies on the responses of T-cell deprived mice to these conjugates have indicated that they are, in fact, T-dependent (Aird 1971; Rubin et al. 1973). It has been suggested that T-cells can recognise the NAD even though B-cells cannot do so.

There is considerable evidence from studies in T-deprived mice that B-cell memory generation is independent of T-cell help (see Chapters 1 and 3), although there is some evidence to the contrary (Schlegel 1974). The expression of memory in a 2° response is, however, highly T-cell dependent. This is consistent with observations that memory development for antibody-mediated cytotoxicity is independent of epitope density, but can only be detected following challenge with a low density.
conjugate (Vachek & Kolsh 1975). However Klaus and Cross (1974) showed that DNP<sub>50</sub>BSA was a poor inducer of memory B-cells and that the response following challenge with DNP-MSH was low, even with provision of large numbers of MSH primed T-cells. In contrast DNP<sub>5</sub>BSA produced an excellent anti-DNP memory cell population. This would imply that T-cell recognition and help is required for B-cell priming.

**Hapten-RBC conjugates**

Hapten-RBC conjugates have often been used in PFC and haemagglutination (HA) assays for detection of anti-hapten responses raised to hapten-protein conjugates. DNP-HRBC (Okumura & Tada 1973), TNP-SRBC (Kettman & Dutton 1970; Naor et al. 1974), NNP-SRBC and NNP-HRBC (Cheers & Miller 1972) have also been used for immunisation, injected intraperitoneally in similar doses to those used for RBC alone. Results have been a little difficult to correlate with one another, partly due to the uncertainty about epitope density.

Two methods are available for the conjugation of DNP to RBC. One entails covalent attachment of the hapten to the RBC and the other attachment of the hapten to the RBC surface by means of an intermediate protein carrier. The first is technically more simple but often yields fragile RBC at high epitope densities, and RBC too insensitive for a PFC assay at low epitope densities (Havas & Hraba 1969; Layson & Sehon 1967) though the method has proved useful in HA assays (Ling 1961; Bullock & Kantor 1965). Trump (1972) has succeeded in producing relatively stable DNP-SRBC conjugates, sensitive in a PFC assay. He used DNFB (dinitofluorobenzene) for conjugation, and believes that the
failure of others with this method may have been due to hapten inhibition of PFC development by free hapten. His results compare well with those of others using an intermediate protein molecule such as BSA (Yamada & Yamada 1969) or rabbit anti-DRBC fragments (Feldmann & Basten 1971) for dinitrophenylation of RBC. The method of Trump (1972) was chosen for the following study.

Most groups have used SRBC or HRBC for their carrier RBC (Kettman & Dutton 1970; Cheers & Miller 1972; Trump 1972; Okumura & Tada 1973; Naor et al 1974). It is essential when immunising with the hapten conjugated to one RBC and assaying with it conjugated to another, that the two RBCs do not cross-react. This problem is sometimes circumvented by plaqueing against the second RBC alone, and subtracting these PFC from those seen after plaqueing against the hapten-RBC conjugate. Cross-reactivity between RBCs has been studied at both the B and T cell level. Cross-reactivity is seldom seen at the B-cell level although SRBC and goat RBC do induce some cross-reactive antibodies (Hoffman & Kappler 1973) and under certain conditions SRBC and cow RBC can produce PFC capable of lysing either type of RBC (Cunningham & Sercarz 1971). Pig, rabbit, burro and chicken RBC do not show any B-cell cross-reactivity with SRBC. At the T-cell level, cross-reactivity appears to be more extensive. This has usually been shown by either priming T-cells with one RBC and testing their ability to help bone marrow cells in an adoptive response to another (Playfair & Marshall-Clarke 1973), or by priming spleen cells and testing their ability to help in an anti-hapten response (Falkoff & Kettman 1972; Hoffman & Kappler 1973). Pig and rabbit RBC showed a fairly marked T-cell cross-reactivity.
with SRBC, burro RBC showed slight cross-reactivity and chicken RBC (CRBC) showed none. Phursh & Chan (1976) also failed to demonstrate T-cell cross-reactivity between CRBC and SRBC but found 13-20% cross-reactivity between burro or horse RBC and SRBC. They did this by comparing the cross-reactions between specifically-localising cells generated in response to SRBC with those generated to other RBC. It is believed, though not yet formally proved, that this is a measure of T-cell localisation. SRBC and CRBC thus seemed the ideal RBC pair for the work described in this chapter.

**Aim of study**

The hapten DNP has been chosen for these studies because it has been extensively used and should permit useful comparisons with the results of many other groups. The aim of the work described in this chapter has been to establish a sensitive assay for anti-DNP antibodies and to determine good immunisation schedules for primary and secondary responses to DNP-protein and RBC conjugates. In addition, experiments are described in which the carrier effect (Bullock & Möller 1974) was investigated, particularly with respect to DNP-CRBC, since T and B cell responses can be distinguished by this means.
MATERIALS & METHODS

1. ANIMALS

Mice of the inbred strain CBA/H were used for these studies. 3-5 month old animals of the same sex were usually used, though X-irradiated mice were sometimes up to 1 year old.

2. ANTIGENS AND IMMUNISATION

Sheep, Chicken and Donkey Erythrocytes (SRBC, CRBC and DRBC)

These were obtained in sterile Alsever's medium from Tissue Culture Services Ltd., Slough, England. They were washed at least three times in isotonic saline and injected in one of the following ways:

a) intraperitoneally (ip): $5 \times 10^6$ or $5 \times 10^7$ RBC in 0.2 ml sterile physiological saline.

b) subcutaneously (sc): $5 \times 10^6$ or $5 \times 10^7$ RBC in 0.1 ml saline evenly distributed between two sites - either the two front, or two hind, footpads.

Dinitrophenyl-protein Conjugates

Dinitrophenyl-bovine γ-globulin (DNP-BGG) and dinitrophenyl-human serum albumin (DNP-HSA) were obtained from Calbiochem (Herts.). The conjugation ratios were 45 moles DNP/mole BGG and 36 moles DNP/mole HSA. Immunisation was by one of the following routes:

a) sc: 50μg DNP-BGG or DNP-HSA in 0.1 ml saline evenly divided between either the two front or two hind footpads, or the same procedure but incorporating $5 \times 10^8$ heat killed Bordetella pertussis (Bp) organisms (Burrough's Wellcome Ltd.).

b) ip: 50μg DNP-protein + $2 \times 10^9$ B. pertussis organisms in 0.2 ml saline, or a 1:1 mixture of DNP-protein in Freund's complete Adjuvant.
(PCA) (Difco, Surrey) mixed in a Sorval omnimixer and injected in 0.4 ml.

**DNP-RBC Conjugates**

Preliminary experiments were conducted coupling DNP to SRBC using difluorodinitrobenzene (FDNFB) (BDH Chemicals, Dorset) (method of Bullock & Kantor 1965). However at high conjugation ratios DNP-SRBC prepared by this method were unstable and lysed in a PFC (plaque forming cell) assay. At lower conjugation ratios PFC results were highly variable between experiments indicating unreliability in the degree of conjugation. Throughout most of this work fluorodinitrobenzene (DNFB) (Sigma Chemical Co., London) has been used according to the method of Trump (1972). This method yielded DNP-SRBC which were stable in a PFC assay forming large, clear plaques similar to those formed in an anti-SRBC response. Despite the evident stability of these DNP-SRBC when incubated alone, incubation with certain batches of guinea-pig complement made them extremely unstable. Guinea-pig complement was routinely absorbed against SRBC but not against DNP-SRBC as the DNP tended to dissociate from the red cell membrane under these conditions. Thus it is possible that anti-DNP activity in the guinea-pig serum was responsible for the cell lysis. In an attempt to remove anti-DNP antibodies, complement was absorbed against DNP-sepharose (Calbiochem) but this had little effect on the stability of DNP-SRBC so it remains possible that the cells were merely fragile to certain sera. As a consequence, PFC responses were routinely counted on the day of assay, before lysis became extensive, and this gave extremely consistent results between experiments.

**Coupling procedure** (Trump 1972)

CRBC or SRBC were washed three times in physiological saline,
then 1 ml packed SRBC was added to 10 ml TRIS buffer pH 8.0 (Appendix) in a 100 ml beaker. All constituents were at room temperature throughout and the beaker was shielded from the light by wrapping in tin-foil. Various volumes of stock DNFB solution (0.4 ml DNFB in 1.0 ml acetone prepared immediately before use) were added dropwise and the reaction allowed to proceed for 20 minutes. After this time the reaction was terminated by addition of 10.0 ml Tris-glycyl-glycine pH 8.0 (Tris-gly-gly. Appendix), and transferred to a centrifuge tube by forcibly ejecting through a pasteur pipette. At high DNFB concentrations this procedure causes any untreated DNFB to sediment out, thus reducing the chances of free hapten remaining in the mixture. All but 0.5 ml was transferred to a 50 ml centrifuge tube, a further 25 ml Tris-gly-gly was added, and then spun down at 4°C. An intensely yellow supernatant formed and there was little or no lysis of the RBC. These conjugated RBC were then washed three times in 40 ml amounts of TRIS + glucose + BSA buffer pH 7.4 (TRIS + D + BSA - Appendix).

DNF-RBC were diluted in TRIS + D + BSA buffer and injected sc or ip with/without B. pertussis, as for unconjugated RBC.

3. CELL SUSPENSIONS

Spleen or lymph nodes were gently dissociated using a ground glass homogeniser, seived through stainless steel screens and washed once (spleens) or twice (lymph nodes). Cell suspensions for the PFC assay were prepared in Dutton's balanced saline (DBS - Appendix).

4. PLAQUE ASSAY (PFC assay)

A modification of the haemolytic plaque assay (Jerne & Nordin 1963) was employed. Agarose (L'Industrie Biologique Francaise, Genevilliers, France) in DBS was used for both top and bottom cell supporting layers. IgG PFC were detected by inclusion in the top
layer, of a rabbit anti-mouse total IgG anti-serum diluted 1:1000 in DBS. The developing constant of this anti-serum was 1.73 (Kd) and the inhibition constant was 0.88 (Ki). Results were expressed as \( \log_{10} \text{PFC} \) per organ assayed. Guinea-pig complement was diluted 1:10 in DBS. DNP-RBC were prepared immediately before use and diluted to 20% in TRIS + D + BSA buffer.

5. **STATISTICS**

Student's 't' test was used throughout this work.

6. **ISOELECTRIC FOCUSING ASSAY (IEF assay)**

This assay was performed according to the method given in Chapter 3.

Due to the instability of DNP-SRBC in the presence of complement, detection of bands by lysis of RBC incorporated in the overlay proved unsuccessful. Visualisation of focused antibodies by reaction with radiolabelled DNP-HSA (method of Keck et al 1973) proved successful. Protein bands were immobilised in the gel, after focusing, by treatment with glutaraldehyde after soaking with 18% sodium sulphate solution. Antibody bands were located by overnight treatment of the plates with 50\( \mu \)Ci of \(^{125}\text{I}-\text{HSA}-\text{DNP}\). After drying, the plates were placed in contact with Kodak No-Screen Medical Film in an X-ray exposure holder and after 1-3 days developed with Kodak X-ray Developer.
I Assay of the anti-DNP response

Preparation of DNP-SRBC for use in a PFC assay

Two methods of preparing DNP-SRBC were compared, that of Trump (1972) using DNFB and that of Bullock and Kantor (1965) using FDNFB. Trump (1972) reported high numbers of anti-DNP SRBC using SRBC conjugated with 32.8 mg DNFB/ml packed RBC and suggested that his consistent good results were due to efficient removal of unreacted hapten. In the present experiments it was first found that coupling with 16.4 mg, 32.8 mg, 41.0 mg and 82 mg DNFB/ml packed SRBC gave stable DNP-SRBC after overnight incubation in the cold. However only those coupled with 16.4 mg were stable for 48 hours or during overnight incubation on an agar plate, so these were tested for sensitivity in a PFC assay.

Mice were immunised ip with 50μg DNP-BGG in FCA and boosted four days later with DNP-BGG in saline. A response of 743 direct PFC/10^6 spleen cells was detected with an FCA background response of 23 PFC/10^6 cells. This is similar in magnitude to the response obtained in some preliminary experiments using DNP-SRBC prepared by the alternative method of Bullock and Kantor (1965). However it was found that their technique, using FDNFB, gave highly unstable cells at epitope densities sufficiently high to be sensitive in a PFC assay. In addition high background levels of PFC were often found. In contrast, the PFC assayed by the method of Trump (1972) were usually small and sharp, and background numbers were always low. In all other experiments this same concentration of DNFB (16.4 mg) was used for the preparation of DNP-SRBC for use in the PFC assay.
There is no accurate way to determine the DNP density on the SRBC, so it has become customary to express the relative degrees of conjugation arbitrarily by the concentration of DNFB or FDNFB added to the reaction mixture. Throughout this thesis, this will be expressed, not as the concentration of DNFB added to 1 ml packed RBC, but as the volume of stock DNFB in acetone reacted with 1 ml packed RBC (Table I).

**Specificity of anti-DNP PFC**

The specificity of anti-DNP PFC was tested in a PFC inhibition assay by addition of DNP-HSA to the overlay. Three different immunisation regimes were used and in each case responses were inhibited 70-80% by a DNP concentration of $5.75 \times 10^{-9}$M (Table II).

**Comparison of different DNP-RBC conjugates in a PFC assay**

Cell suspensions from DNP-ESG or DNP-RBC immunised mice were assayed against DNP$_{0.04}$SRBC, DNP$_{0.04}$CRBC and DNP$_{0.04}$DRBC. The DNP$_{0.04}$DRBC were very unstable and thus unsuccessful for use in a PFC assay. A ten-fold lower conjugation ratio (DNP$_{0.02}$DRBC) proved insensitive for detection of PFC. DNP$_{0.04}$CRBC were stable and sensitive but detected a higher anti-DNP background than did DNP$_{0.04}$SRBC (Table III). CRBC plaques were also less well-defined than SRBC plaques so it was decided to continue using DNP$_{0.04}$SRBC for assay wherever possible.

**Immunisation with DNP-RBC**

Mice were immunised with heavily conjugated DNP$_{0.2}$CRBC or DNP$_{0.2}$SRBC by sc injection of $5 \times 10^7$ cells without adjuvant, divided between the two front footpads. The DNP-RBC were prepared immediately prior to use as DNP was slowly lost into solution.

Cell suspensions prepared from the brachial lymph nodes, which show
### TABLE I

**NOMENCLATURE FOR DNP-RBC**

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration of DNFB/ml packed RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNP₀.₀₂ RBC</td>
<td>8.2 mg/ml</td>
</tr>
<tr>
<td>DNP₀.₀₄ RBC</td>
<td>16.4 mg/ml</td>
</tr>
<tr>
<td>DNP₀.₀₈ RBC</td>
<td>32.8 mg/ml</td>
</tr>
<tr>
<td>DNP₀.₁ RBC</td>
<td>41.0 mg/ml</td>
</tr>
<tr>
<td>DNP₀.₂ RBC</td>
<td>82.0 mg/ml</td>
</tr>
</tbody>
</table>

Preparation of DNP-RBC by covalent coupling with DNFB according to the method of Trump (1972).
### TABLE II

**INHIBITION OF ANTI-DNP PLAQUES BY DNP-HSA**

<table>
<thead>
<tr>
<th>Immunisation</th>
<th>Mean (arithmetic) direct PFC/plate</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Inhibited</td>
</tr>
<tr>
<td>a) DNP-BGG in CFA ip (d0)</td>
<td>354.5</td>
<td>43.0</td>
</tr>
<tr>
<td>DNP-BGG in saline (d4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b) DNP-BGG + B. pertussis sc</td>
<td>23.5</td>
<td>6.6</td>
</tr>
<tr>
<td>c) DNP₀.₂ CRBC + B. pertussis sc</td>
<td>54.0</td>
<td>13.2</td>
</tr>
</tbody>
</table>

Day 7 PFC responses vs DNP₀.₄ SRBC
(a) 4 mice/group; (b) and (c) 5 mice/group.

DNP-HSA incorporated in the agarose overlay at a DNP-concentration of 5.75 x 10⁻⁹ M.
<table>
<thead>
<tr>
<th>Immunisation</th>
<th>Mean $\log_{10}$ IgM PFC ± s.e.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNP 0.04 SRBC</td>
</tr>
<tr>
<td>Normal background</td>
<td>2.44 ± 0.19</td>
</tr>
<tr>
<td>(spleen)</td>
<td></td>
</tr>
<tr>
<td>FCA background</td>
<td>2.37 ± 0.12</td>
</tr>
<tr>
<td>(spleen)</td>
<td></td>
</tr>
<tr>
<td>DNP-BSG in FCA d0</td>
<td>4.58 ± 0.10</td>
</tr>
<tr>
<td>DNP-BSG in saline d4</td>
<td></td>
</tr>
</tbody>
</table>

Day 7 PFC responses of groups of 5 mice immunised with 50μg DNP-BSG ip in Freund's complete adjuvant (FCA) and boosted 4 days later with DNP-BSG 50μg DNP/ip in saline. Control mice were either untreated, or received FCA ip only.
## Table IV

**Immunisation with DNP-RBC**

<table>
<thead>
<tr>
<th>Immunisation</th>
<th>Log$_{10}$ PFC/4 Brachial Lymph Nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNP$_{0.04}$ CRBC</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
</tr>
<tr>
<td>-</td>
<td>0.66</td>
</tr>
<tr>
<td>(4.57) (3.98)</td>
<td></td>
</tr>
<tr>
<td>5 x 10$^7$ DNP$_{0.2}$ CRBC sc</td>
<td>3.75</td>
</tr>
<tr>
<td>(5623) (21380)</td>
<td></td>
</tr>
<tr>
<td>5 x 10$^7$ DNP$_{0.2}$ SRBC sc</td>
<td>2.40</td>
</tr>
<tr>
<td>(251) (239)</td>
<td></td>
</tr>
</tbody>
</table>

Brachial lymph nodes from 2 mice were pooled for a day 5 PFC assay against DNP$_{0.04}$ CRBC or DNP$_{0.04}$ SRBC. Results are expressed as the log$_{10}$ mean (or anti-log of the mean) of duplicate cell suspensions.
Fig. 2.1 Autoradiograph of electrofocused anti-DNP antibodies.

IEF spectra of day 7 sera of irradiated (900r) recipients of $2 \times 10^7$ (A and B), or $10^7$ (E), brachial and axillary lymph node cells and $10^8$ DNP-0,2 CRBCiv. Donors were primed 6 weeks before transfer with $5 \times 10^7$ DNP-0,2 CRBCsc + $5 \times 10^8$ B.pertussis sc. Group B were boosted sc with $5 \times 10^7$ DNP-0,2 CRBC in saline 1 day before transfer.

C and D, primed with 50µg DNP-HSA + $5 \times 10^8$ B.pertussis and challenged 6 weeks later with 50µg DNP-HSA in saline. They were bled on day 7(C) or 17(D).
low background levels of PFC, were plaqued against \( \text{DNP}_{0.04} \text{CRBC} \) or \( \text{DNP}_{0.04} \text{SRBC} \). Results are shown in Table IV. It is clear that immunisation with \( \text{DNP}_{0.2} \text{CRBC} \) induces a good anti-DNP response (as measured by a PFC assay against \( \text{DNP}_{0.04} \text{SRBC} \)) and a good anti-CRBC response (as measured by subtraction of PFC detected against \( \text{DNP}_{0.04} \text{SRBC} \) from those detected against \( \text{DNP}_{0.04} \text{CRBC} \)). Similar results were seen after immunisation with \( \text{DNP}_{0.2} \text{SRBC} \), good anti-DNP and anti-SRBC PFC responses being detected. The lower anti-DNP response seen after immunising with \( \text{DNP}_{0.2} \text{SRBC} \), compared with that after \( \text{DNP}_{0.2} \text{CRBC} \), may reflect either less efficient immunisation by \( \text{DNP}_{0.2} \text{SRBC} \) or a less sensitive PFC assay by \( \text{DNP}_{0.04} \text{CRBC} \).

### Isoelectric focusing of anti-DNP antibodies

The instability of \( \text{DNP}_{0.04} \text{SRBC} \) in the presence of guinea-pig complement made it impossible to incorporate these red cells in an agarose overlay for detection of electrofocused anti-DNP antibodies. Occasionally a few bands were detected with good batches of complement but usually lysis occurred too rapidly. However, preliminary experiments using the method of Keck et al. (1973) for autoradiographic detection of electrofocussed anti-DNP antibodies, have proved extremely sensitive (Fig. 2.1). This technique was not developed in time for routine use in the work described in Chapters 3 and 6.

### II Primary (1\textsuperscript{st}) response to DNP

#### Immunisation with DNP-CRBC

Mice were immunised sc or ip with either \( \text{DNP}_{0.2} \text{CRBC} \) or CRBC alone, for assay of both the anti-DNP and anti-CRBC PFC responses in the lymph nodes and spleen. The results are shown in Fig. 2.2. A good IgM and IgG PFC response was seen following both ip and sc immunisation with \( \text{DNP}_{0.2} \text{CRBC} \). CRBC alone gave no significant
Fig. 2.2 Immunisation with DNP$_2$CRBC.

Day 7 anti-DNP(A) and anti-CRBC(B) PFC responses in groups of 5 mice immunised with:

a, DNP$_2$CRBC; b, CRBC; c, untreated.

1, immunisation with $5 \times 10^8$ RBC ip for assay of spleens.

2, immunisation with $5 \times 10^7$ RBC sc for assay of brachial lymph nodes.

| IgM PFC | IgG PFC |
Fig. 2.3 Kinetics of the 1° response to DNP$_{0.2}$CRBC.

Groups of 5 mice were immunised with 5x10$^7$ DNP$_{0.2}$CRBC sc in the two front(a) or hind(b) footpads. PFC assay vs DNP$_{0.04}$SRBC.

- - - - - IgM PFC per brachial lymph node pair;

- - - - - IgG PFC per brachial lymph node pair.
a. Brachial

mean log_{10} PFC+se

Day of assay

b. Popliteal

mean log_{10} PFC+se

Day of assay
Fig. 2.4 Effect of epitope density on the $1^0$ response to DNP-CRBC. Anti-DNP PFC responses in groups of 5 mice immunised sc in the two front footpads with (a) $5 \times 10^7$ DNP$_{0.2}$CRBC or (b) $5 \times 10^7$ DNP$_{0.02}$CRBC.

■■■■■, IgM PFC per brachial lymph node pair;

●●●●●, IgG PFC per brachial lymph node pair.
Fig. 2.5 Effect of B. pertussis on the $^{1}_0$ response to DNP$_0.2$ CRBCsc.

Anti-DNP PFC response in groups of 5 mice immunised sc in the front footpads with (a) $5 \times 10^7$ DNP$_0.2$ CRBC alone or (b) $5 \times 10^7$ DNP$_0.2$ CRBC + $5 \times 10^8$ B. pertussis.

- - - - - - - - - - - - , IgM PFC per brachial lymph node pair;
- - - - - - - - - - - - - - - - - , IgG PFC per brachial lymph node pair.
increase in IgG anti-DNP plaques, though an increase in IgM PFC was seen after sc immunisation. The anti-CRBC IgM PFC response was studied following immunisation with both CRBC and DNP\textsubscript{0.2}CRBC. Similar levels of PFC were found after ip immunisation, while CRBC alone was slightly more efficient at raising the anti-CRBC response by the subcutaneous route. It is clear that DNP epitope density was not so high as to mask native CRBC determinants and thereby prevent the induction of an anti-carrier response.

The kinetics of a 1\textsuperscript{o} response to DNP\textsubscript{0.2}CRBC were followed after immunising mice in either the front, or hind, footpads with $5 \times 10^7$ DNP\textsubscript{0.2}CRBC sc. The results of day 3, 5 and 7 PFC assays are shown in Fig. 2.3. IgM PFC were detected in a day 3 assay and rose in number to give a peak at day 5. IgG PFC were first detected in a day 5 assay of brachial lymph node cells and numbers fell slightly by day 7. However IgG PFC were never detected in a day 5 assay of popliteal lymph node cells.

Fig. 2.4 shows the effect on the 1\textsuperscript{o} anti-DNP response of immunising with the less heavily dinitrophenylated conjugate, DNP\textsubscript{0.02}CRBC. Both the day 3 and 5 IgM PFC responses were lower than after immunising with DNP\textsubscript{0.2}CRBC. The IgG PFC results were poor after both in this experiment. Incorporation of the adjuvant, B. pertussis, with the immunising injection of $5 \times 10^7$ DNP\textsubscript{0.2}CRBC sc did not alter the kinetics of the 1\textsuperscript{o} anti-DNP response but raised the day 5 IgM and day 7 IgM and IgG PFC responses approximately 5 fold (Fig. 2.5).

Immunisation with DNP-BGG

Mice were immunised in the front footpads with 50μg DNP-BGG with or without $5 \times 10^8$ B. pertussis. No response, or a very low
Fig. 2.6 Kinetics of the 1<sup>st</sup> response to DNP-BGG.

Anti-DNP responses in groups of 5 mice immunised sc in the front footpads with 50μg DNP-BGG alone (a and c) or with <i>E. pertussis</i> (b and d).

-·····-, IgM PFC per brachial lymph node pair;
-····, IgG PFC per brachial lymph node pair.
IgM response, was obtained without adjuvant (Fig. 26). When *B. pertussis* was included the IgM anti-DNP PFC response was very similar to that induced by $5 \times 10^7$ DNP$_{0.2}$CRBC sc. IgG PFC were first detected on day 7, a little later than in the brachial lymph node response to DNP$_{0.2}$CRBC.

III Secondary (2°) response to DNP

It has frequently been observed that low RBC doses prime well for a 2° response (Falkoff & Kettman 1972; Cheers & Miller 1972; Black & Inchley 1974). On the other hand, for hapten-protein conjugates, epitope density rather than antigen concentration may determine the degree of priming, and high density conjugates are sometimes poor at inducing good B-cell memory (Klaus & Cross 1974). A comparison was therefore made of the degree of anti-DNP memory elicited by $10^6$ and $5 \times 10^7$ DNP$_{0.2}$CRBC and also of that elicited by high (DNP$_{0.2}$CRBC) and low (DNP$_{0.02}$CRBC) epitope density conjugates (Figs. 27 and 8). In each instance challenge was with $5 \times 10^7$ DNP-CRBC.

A more prompt 2° IgM and IgG PFC response was seen after priming with $5 \times 10^7$ than with $10^6$ DNP$_{0.2}$CRBC, there being higher levels of each on day 3 but equal levels by day 5 (Fig. 2.7). In contrast epitope density had little effect on priming since priming and challenge with $5 \times 10^7$ DNP$_{0.2}$CRBC yielded almost identical IgM and IgG PFC responses to those seen after priming and challenge with $5 \times 10^7$ DNP$_{0.02}$CRBC. Challenge with the opposite conjugate to that used for priming induced a poorer 2° response than did challenge with the same conjugate, this being particularly noticeable after DNP$_{0.02}$CRBC priming and DNP$_{0.2}$CRBC challenge when the 2° response was more transient.

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Fig. 2.7 Priming with DNP₀.₂CRBC: Effect of antigen dose.

Day 3 and 5 \(2^0\) anti-DNP PFC responses in groups of 5 mice primed in the front footpads with \(10^6(\downarrow\downarrow\downarrow)\) or \(5\times10^7(■■■)\) DNP₀.₂CRBC sc, and challenged 5 weeks later with \(5\times10^7\) DNP₀.₂CRBCsc.

●·······●, \(1^0\) response.
a. IgM  
b. IgG

Day of assay

mean $\log_{10}$ PFC $\pm$ se
Fig. 2.8 Effect of epitope density on responses to DNP-CRBC.

Day 3 and 5 anti-DNP PFC responses in groups of 5 mice primed with $5 \times 10^7$ DNP$_{0.2}$ CRBC + $5 \times 10^8$ B.pertussis (c and d), or $5 \times 10^7$ DNP$_{0.02}$ CRBC + $5 \times 10^8$ B.pertussis (e and f).

c and e, challenged with $5 \times 10^7$ DNP$_{0.2}$ CRBC 5 weeks after priming;
d and f, challenged with $5 \times 10^7$ DNP$_{0.02}$ CRBC 5 weeks after priming.

Residual $1^0$ responses at 5 weeks are shown as single lines.

- - - - , IgM PFC response per brachial lymph node pair;
• --•, IgG PFC response per brachial lymph node pair.
Fig. 2.9 The $2^0$ response to DNP-BGG and DNP-HSA.

Day 3 and 5 PFC responses in groups of 5 mice primed in the front footpads with (a) $50\mu g$ DNP-BGG + $5 \times 10^8$ \textit{B. pertussis} or (b) $50\mu g$ DNP-HSA + $5 \times 10^8$ \textit{B. pertussis}, and challenged 3 weeks later with $50\mu g$ of the same DNP-protein in saline. Residual $1^0$ responses are shown as single lines.

■■■■■, IgM PFC per brachial lymph node pair;

●●●●●, IgG PFC per brachial lymph node pair.
El (C) a,

Ct (C) a,

mean log_{10} PFC±se

Day of assay

a) DNP-BGG

b) DNP-HSA
In addition it should be noted that by the time of challenge at 5 weeks, the residual 1° responses were low.

Since a high dose of the higher density conjugate proved best for priming, $5 \times 10^7 \text{DNP}_{0.2} \text{CRBC}$ were used for priming in subsequent experiments with/without adjuvant, followed by challenge with the same dose of the same conjugate without adjuvant.

Very similar kinetics to those seen for DNP-CRBC 2° responses were also seen for the 2° responses to DNP-BGG and DNP-HSA following priming with $50\mu g \text{DNP-protein plus } \text{B. pertussis sc}$, and challenge with the same dose in saline (Fig. 2.9). IgG PFC were high at day 3 and either remained equal or rose slightly by day 5. IgM PFC responses to DNP-BGG fell between days 3 and 5 while those to DNP-HSA continued to rise. The most striking difference between these and the DNP-RBC responses was the 5-10 fold lower 2° IgM response despite the similarity in 2° IgG levels. It was also noted that although 1° antibody production was still evident at 3 weeks after priming (Fig. 2.9) the levels were low at this time.

IV The effect of carrier priming on the 1° anti-DNP response

Carrier priming for a response to DNP-CRBC

The effect of priming with $10^6 \text{CRBC plus } \text{B. pertussis}$ on the 1° anti-DNP response to $5 \times 10^7 \text{DNP}_{0.2} \text{CRBC}$, using fore or hind footpads, is shown in Fig. 2.10. The IgM PFC response in both experiments followed the pattern of the 2° IgM response, there being identical PFC numbers in the experimental and 2° control group with a plateau between days 3 and 5. In contrast 1° IgM PFC showed a clear peak at day 5. Prior injection of CRBC did not result in an 1° IgG response to DNP upon challenge with $\text{DNP}_{0.2} \text{CRBC}$ and, in fact, appeared to suppress the day 5 1° IgG.
**Fig. 2.10** Effect of carrier priming on the $^{10}$ response to DNP;
response to DNP$_{0.2}$CRBC.

A, front footpads.

B, hind footpads.

(a) $^{10}$ response to $5 \times 10^7$ DNP$_{0.2}$CRBC sc.

(b) $^{20}$ response to $5 \times 10^7$ DNP$_{0.2}$CRBC sc. Mice were primed with
$5 \times 10^7$ DNP$_{0.2}$CRBC + $5 \times 10^8$ B.pertussis 5 weeks before challenge.

(c) Mice were primed with $10^6$ CRBC + $5 \times 10^8$ B.pertussis sc 5 weeks
before immunisation with $5 \times 10^7$ DNP$_{0.2}$CRBC sc.

•••••, IgM PFC per brachial lymph node pair;

•••••, IgG PFC per brachial lymph node pair.
Fig. 2.11 Effect of carrier priming on the $1^0$ response to DNP:

responses to DNP-BGG and DNP-HSA.

(a) $1^0$ response to 50µg DNP-protein injected sc in saline.

(b) $2^0$ response to 50µg DNP-protein. Mice were primed with 50µg DNP-BGG or DNP-HSA + 5x10^8 B.pertussis, and challenged 5 weeks later with 50µg of the respective DNP-protein in saline.

(c) Mice were primed with 50µg BGG or HSA + 5x10^8 B.pertussis 5 weeks before immunisation with 50µg DNP-protein.

-·····-, IgM PFC per brachial lymph node pair;

-———·-, IgG PFC per brachial lymph node pair.
normally found in the brachial nodes (Fig. 2.10a).

**Carrier priming for responses to DNP-BGG and DNP-HSA**

The effect of carrier priming on the 1\(^{o}\) anti-DNP response to 50μg DNP-BGG or DNP-HSA injected into the two hind footpads is shown in Fig. 2.11. The IgM PFC response was very slightly raised above 1\(^{o}\) levels, particularly in the DNP-HSA response (2.11-2c) but remained below that of the control 2\(^{o}\) IgM response. No significant increase in IgG production was detected in any of the carrier-primed groups.

V The 2\(^{o}\) response to DNP after challenge with a different DNP-conjugate to that used for priming

Figs. 2.12 and 2.13 show the results of experiments involving 50μg DNP-BGG or DNP-HSA plus 5 x 10\(^{8}\) B. pertussis, and challenge 3 or 5 weeks later with 50μg DNP-BGG, 50μg DNP-HSA or 5 x 10\(^{7}\) DNP\(_{0.2}\) CRBC sc. In each experiment, a good 2\(^{o}\) IgG response was obtained upon challenge with the same conjugate as that used for priming. Challenge with a heterologous conjugate resulted in a delayed 2\(^{o}\) IgG PFC response which usually reached similar levels at day 5 to those produced by the homologous conjugate. An exception followed priming with DNP-BGG and challenge with DNP-HSA when no 2\(^{o}\) response was obtained at all (Fig. 2.12-1b). It is possible that the immunogenicity of the carrier use for challenge is important here. This will be considered in more detail in the discussion. It is interesting to note that challenge with DNP\(_{0.2}\) CRBC (Fig. 2.13) raised the IgM PFC response above the level of that in the 2\(^{o}\) responses to DNP-protein.

It seemed likely that the delay in the 2\(^{o}\) IgG PFC response after challenge with the heterologous conjugate compared with the
Fig. 2.12  The "carrier effect"

Day 3 and 5 2^0 PFC responses in groups of 5 mice.

Expt. 1; mice were primed sc in the front footpads with 50µg 
DNP-BGG + 5x10^8 B. pertussis and challenged 5 weeks later 
with (a)50µg DNP-BGG or (b)50µg DNP-HSA.

Expt. 2; mice were primed sc in the front footpads with 50µg 
DNP-HSA + 5x10^8 B. pertussis and challenged 5 weeks 
later with (a)50µg DNP-HSA or (b)50µg DNP-BGG.

■■■■, IgM PFC per brachial lymph node pair;

•—•–, IgG PFC per brachial lymph node pair.
Fig. 2.13 The "carrier effect"

Groups of 5 mice were primed with (1) 50µg DNP-BGG + 5x10⁸ B. pertussis or (2) 50µg DNP-HSA + 5x10⁸ B. pertussis, sc and challenged 5 weeks later with (a) 50µg of the same DNP-protein as used for priming, or (b) 5x10⁷ DNP₀.₂ CRBC sc.

- - - - - , IgM PFC per brachial lymph node pair;
• - - - - , IgG PFC per brachial lymph node pair.
Day of assay

1a.

1b.

2a.

2b.

mean $\log_{10}$ PFC±se

Day of assay

3 5
Fig. 2.14 The "carrier effect"

1,2. Duplicate experiments.

Groups of 5 mice were primed with (a) 50μg DNP-BGG + 10^6 CRBC + 5x10^8 B.pertussis sc, or (b) 50μg DNP-BGG + 5x10^8 B.pertussis sc and challenged 5 weeks later with 5x10^7 DNP_0.2 CRBC sc.

- ....... , IgM PFC per brachial lymph node pair;
- ...... , IgG PFC per brachial lymph node pair.
prompt response after challenge with the homologous conjugate reflected differences in the kinetics of primed and virgin helper to T-cells. For this reason it was decided to prime mice with DNP-BCG plus CRBC for challenge 5 weeks later with DNP-CRBC. This procedure ensures the presence of both-hapten-specific and carrier specific memory cells although they were not primed by the same conjugate. PFC results can be seen in Fig. 2.14. Supplemental CRBC priming with DNP-BCG did indeed raise the day 3 IgG PFC responses to DNP \textsuperscript{0.2} CRBC above the level of those in mice primed with DNP-BCG alone.
Assay of the anti-DNP response with DNP-RBC

A comparison of SRBC dinitrophenylation by the methods of Bullock and Kantor (1965) and Trump (1972) indicated that FDNPB was a less effective haptentising agent than DNFB. DNP-SRBC, prepared by reacting SRBC with FDNPB, were either unstable or insensitive in a PFC assay. In contrast, DNP$_{0.04}$SRBC formed by reacting 16.4µg DNFB with 1 ml packed SRBC were consistently stable and sensitive for assay of anti-DNP PFC responses. In addition, plaques were usually small, sharp and easy to count. Specificity was demonstrated by a plaque inhibition assay using DNP-HSA.

In order to compare the sensitivity of these DNP$_{0.04}$SRBC with that of the DNP-SRBC prepared by Trump (1972), mice were immunised with DNP$_{45}$RGG following a similar régime to that used with DNP$_{36}$RGG, although a 10 fold lower dose was injected. A direct anti-DNP PFC response of 743 PFC/10$^6$ spleen cells was detected compared with 180 PFC/10$^6$ spleen cells (Trump 1972). Background responses were also similar.

Assay with haptenated DRBC or CRBC was less successful than with SRBC. DNP-DRBC were either unstable or insensitive for detection of anti-DNP PFC. DNP$_{0.04}$CRBC were both stable and sensitive, but CRBC plaques were never sharp and clear, probably because of the large size of the chicken red cell. Despite the stability and sensitivity of both DNP$_{0.04}$SRBC and DNP$_{0.04}$CRBC neither could be used in an IEF assay due to considerable lysis resulting from long incubations (more than 2 hours) with complement. Development of the autoradiographic technique (Keck et al 1973) should be useful in future studies.
Immunisation with DNP-RBC

The choice of DNP-RBC for immunisation in the present work, was restricted to DNP-CRBC due to the instability of heavily conjugated DRBC and the preference for DNP-SRBC for assay. DNP-SRBC were also used as an antigen in preliminary work and the response was assayed against both SRBC and DNP-SRBC. The anti-DNP response could thus be calculated but this procedure proved tedious and was not continued.

Kettman and Button (1970) reported only a weak anti-TNP response following immunisation with lightly haptenated RBC but a good IgM and IgG PFC response to heavily haptenated RBC. Heavily dinitrophenylated CRBC were prepared for immunisation by reacting 82 mg DNP with 1 ml packed CRBC (DNP0.2 CRBC). In preliminary experiments mice were injected intraperitoneally with 5 x 10^8 DNP0.2 CRBC without adjuvant. Both high IgM and IgG anti-DNP PFC responses were detected (Fig. 2.2). The IgM PFC response to the carrier was also studied and compared with that induced by immunisation with CRBC alone. After ip immunisation the anti-CRBC responses to DNP0.2 CRBC and CRBC were identical but after sc immunisation that to DNP0.2 CRBC was slightly lower, perhaps indicating some masking of the carrier molecule by haptenic determinants. There is, however, no indication from these results that DNP0.2 CRBC is a T-independent antigen.

Okumura and Tada (1973) immunised mice with heavily dinitrophenylated HRBC, also prepared by the method of Trump (1972). They injected 5 x 10^8 DNP-HRBC ip with 2 x 10^9 B. pertussis, then boosted with 2.5 x 10^8 DNP-HRBC 5 days later and assayed the response at intervals thereafter. They found optimal direct PFC
responses two days after the second injection, reaching 55,800 direct PFC/spleen. However IgG responses were always poor and IgM responses were low if the adjuvant was omitted. Anti-carrier responses were slightly higher than the anti-DNP responses (61,800 direct PFC/spleen). Reasons for the differences between these results and those of the present work, are uncertain and may be related to choice of carrier, degree of haptenation and the stability of heavily haptenated RBC.

Immunisation by the subcutaneous route was also investigated, and good immunisation regimes developed for use in subsequent work (recorded in Chapter 6). Preliminary experiments (Table IV and Fig. 2.2) indicated that injection of $5 \times 10^7$ DNP$_{0.2}$CRBC sc to the two front footpads induces a moderate IgM and IgG PFC response in the brachial lymph nodes, and that the lymph node anti-DNP background response is low.

**Primary and secondary responses to subcutaneous DNP$_{0.2}$CRBC**

Following sc injection into the respective footpads, the $1^\circ$ IgM PFC response of either the pooled brachial, or pooled popliteal lymph node cells was apparent by day 3 and rose to a peak at day 5. IgG PFC were first detected in the brachial nodes by a day 5 assay, but none were seen in the popliteal nodes at that time (Fig. 2.3). Naor et al (1974) followed the $1^\circ$ anti-TNP PFC response after iv immunisation with heavily haptenated TNP-SRBC. Both IgM and IgG PFC were seen in a day 4 assay but IgG only by day 7. There was no evidence for a more rapid fall in IgM than IgG PFC in the sc response to DNP$_{0.2}$CRBC. Immunisation with the less heavily haptenated conjugate, DNP$_{0.02}$CRBC, induced a poorer IgM PFC response than did

42.
DNP_{0.2}CRBC (Fig. 2.4). These results are in accord with those of Naor et al (1974) showing that heavily conjugated TNP-SRBC were best for induction of an IgM response. Immunisation with DNP_{0.2}CRBC together with 5 \times 10^8 P. pertussis raised PFC levels by about 5 fold on both days 5 and 7 of assay (Fig. 2.5).

In a 5 week 2° response to 5 \times 10^7 DNP_{0.2}CRBC, IgM and IgG PFC levels were high at day 3 and fell slightly by day 5 (Fig. 2.8). Cheers and Miller (1972) primed mice with an intravenous injection of 5 \times 10^8 NNP-HRBC and found high levels of 2° anti-NNP IgM PFC 4-6 days after intravenous challenge with 5 \times 10^8 NNP-HRBC.

The 2° response to DNP was affected by variation of the priming antigen dose, priming being more efficient with 5 \times 10^7 than with 10^6 conjugated RBC. This stands in contrast to studies on low dose priming for 2° responses to SRBC (Falkoff & Kettman 1972; Cheers & Miller 1972; Black & Inchley 1974). Variation in epitope density, on the other hand, did not greatly affect the 2° anti-DNP response.

Both high (DNP_{0.2}CRBC) and low (DNP_{0.02}CRBC) density conjugates primed well for a 2° anti-DNP response but were most effective when challenge was with the same conjugate as that used for priming. This indicates that there may be some differences between the determinants of DNP_{0.2}CRBC and DNP_{0.02}CRBC which are recognised by helper T-cells. Although it has been shown that DNP_{0.2}CRBC contains CRBC determinants which can be recognised by both T and B cells (Fig. 2.2), it is possible that this conjugate contains more NAD than does the low density conjugate. Challenge with the same conjugate as that used for priming should thus stimulate the maximum number of primed T-cells.

**Primary and secondary responses to subcutaneous DNP-protein**
50μg DNP-BGG plus 5x10^8 B. pertussis were injected into the two front footpads and PFC numbers in the draining brachial lymph nodes were determined. IgM PFC peaked between days 5 and 7 while IgG PFC were first seen in a day 7 assay (Fig. 2.6). Both IgM and IgG PFC levels were similar to those seen at the peak of responses to DNP_{0.2} CRBC plus B. pertussis (Fig. 2.5), which is unusual since particulate antigens are generally considered to be better stimulators of the IgM response (Rabin & Rose 1970). Since day 7 IgG production was high, there was no support for this high epitope density conjugate (DNP_{45} BGG) acting as a T-independent antigen. The kinetics of the 1° response compared well with other work using high epitope density, T-dependent conjugates. Havas and Hraba (1969) immunised Balb/c mice sc with 0.1 mg DNP_{40} HGG or DNP_{31} -hemocyanin in incomplete Freund's adjuvant, detected IgM PFC at day 4 and found the response peaked at day 6. It then fell sharply, the PFC response being predominantly IgG by day 8. Likewise Yamada and Yamada (1969) found that IgM PFC peaked at day 5 then fell sharply while the IgG response peaked at day 7 and fell gradually, in response to an ip injection of DNP_{40} HGG. The main difference between both of these experiments and those reported in this work, was the rapid decline in IgM PFC following the peak response. This may be explained by the use of different routes of injection, DNP-conjugates, adjuvants or mice.

2° responses were studied in mice primed with DNP-BGG plus B. pertussis and challenged with DNP-BGG in saline. Both 2° IgM and IgG reached peak PFC numbers within 3 days of challenge. The 2° response to DNP-HSA was slightly slower, day 5 PFC responses...
being higher than those at day 3. However peak levels were similar for both. Havas and Hraba (1969) also reported that the 2° anti-DNP response consisted predominantly of IgG PFC which reached peak numbers four days after challenge.

The carrier effect

Good control 2° responses were obtained to DNP following priming and challenge with the same hapten-carrier conjugate, be it DNP-CRBC, DNP-BGG or DNP-HSA (Figs. 2-8 and 2-9). Mice primed with DNP-BGG and challenged with DNP-CRBC showed a delayed IgG 2° response, lower than the control 2° on day 3 but rising to equal levels by day 5. This delayed 2° IgG response was also seen after challenge of DNP-HSA primed mice with either DNP-CRBC or DNP-BGG. However DNP-HSA challenge of animals primed with DNP-BGG induced only a low response, probably 2° in nature since the day 3 IgG PFC were more numerous than the IgM.

Day 3 IgM PFC responses were similar in mice either primed and challenged with DNP-BGG, or primed with DNP-BGG and challenged with DNP-CRBC. However IgM PFC continued to increase in number in the DNP-CRBC challenged group so that by day 5 the IgM response was higher than in the control 2°. DNP-CRBC challenge following DNP-HSA priming induced higher 2° IgM PFC responses on both days 3 and 5 after challenge. This may either be a property of the DNP-CRBC themselves, or a function of challenge with a heterologous carrier. Since challenge of DNP-HSA primed cells with DNP-BGG did not raise the 2° IgM above control levels, the increased IgM response seen after DNP-CRBC challenge is probably a property of this conjugate.

Successful challenge with hapten-heterologous carrier has often been reported, particularly following high challenge doses,
although responses are frequently lower than those of the normal 2\(^{\text{O}}\) where primed T-cells are also present. Mitchison (1971a) has shown that the effectiveness of a heterologous carrier is dependent on its immunogenicity, chicken γ globulin (CGG) being a far more potent heterologous carrier than bovine serum albumin (BSA) or ovalbumin (OVA). He has derived arbitrary figures for the doses of different antigens which would be required for them to act effectively in a challenge as heterologous carriers, and has compared these doses with those required for challenge as homologous conjugate. For NIP-CGG he estimated that a 30\(\mu\)g challenge as heterologous conjugate would elicit a good anti-NIP response whilst only 0.1\(\mu\)g of the homologous conjugate was required. For BSA, on the other hand, he estimated an 800\(\mu\)g challenge as heterologous conjugate compared with 100\(\mu\)g as homologous conjugate. It is apparent that in situations where no carrier effect could be demonstrated, this may be due to challenge with too high a dose of heterologous carrier conjugate. From this it follows that if BGG behaves similarly to CGG then a challenge of 50\(\mu\)g would be ample to achieve a good 2\(^{\text{O}}\) response to DNP-BGG following DNP-HSA priming. However, if HSA behaves like BSA, then a much higher dose would be required than the 50\(\mu\)g used here. Steiner and Eisen (1967) have reported similar difficulties, resulting from the low immunogenicity of HSA, in priming with DNP-HSA.

The delay in peak IgG PFC levels following challenge with the heterologous carrier may be related to the time required for activation of virgin T-helpers compared with memory T-helpers (Araneo et al 1976). These authors have shown peak helper activity at 48 hours for memory T-cells but not until 72 hours for
virgin T-cells. Experiments to test whether this lag was still found when carrier primed cells were also present are shown in Fig. 2-14. These involved priming with DNP-BGG plus CRBC and challenge with DNP-CRBC, thus providing a source of both hapten and carrier primed cells. In each case the provision of carrier-specific T-cells raised the day 3 IgG PPC levels above those obtained following priming with DNP-BGG alone. This confirms that the potency of an antigen as a heterologous carrier in challenge for an anti-hapten response, is related to its ability to stimulate virgin T-cells to respond and that the time lag is related to the time taken to activate virgin rather than memory T-cells. CRBC was a particularly potent heterologous carrier in the doses used here, BGG was also good but HSA was poor. A higher dose of DNP-HSA may have overcome this.

Carrier pre-immunisation

The effect of CRBC priming alone on the 1° anti-DNP response was investigated by injecting $10^6$ CRBC plus B. pertussis sc 5 weeks prior to immunisation with DNP$_{0.2}$CRBC (Fig. 2-10). In both experiments IgM responses closely resembled those of the 2° rather than the 1° control group. However IgG PFC were totally suppressed by the carrier priming. The effect of prior BGG or HSA treatment on the 1° anti-DNP response after immunisation with DNP-BGG or DNP-HSA, is shown in Fig. 2-11. The similarity of the IgM response to the 2° control group was not as marked in either case as in the DNP$_{0.2}$CRBC system, the response being intermediate in level between the 1° and 2°. No 1° IgG response was detected with or without prior protein carrier priming.

There is abundant evidence, considered in the Introduction to
this chapter, that carrier pre-immunisation augments an anti-hapten response (both IgM and IgG) to a subsequent injection of that hapten-carrier conjugate (Cheers & Miller 1972; Falkoff & Kettman 1972; Kishimoto & Ishizaka 1972; Klaus & Cross 1974). However it is apparent from the experiments presented here that there is not necessarily an augmentation of the $1^{\circ}$ IgM response, but instead a switch from a $1^{\circ}$ to a $2^{\circ}$ type IgM response reflected by raised IgM PFC on certain days of assay but reduced IgM PFC on other days compared with the $1^{\circ}$ control. It has been suggested that IgM memory may not be a property of B-cells but rather a reflection of augmentation of the $1^{\circ}$ IgM response by memory T-cells (Cunningham & Sercarz 1971). Seppala et al (1976) have recently studied the IgM and IgG memory response to NIP-CGG following priming with the cross-reacting DIP-CGG. IgG memory was mainly of anti-NIP specificity indicating the presence of memory B-cells, but IgM memory was largely anti-DIP, implicating the greater importance of T, than B memory cells in this response. However, there is evidence that IgM memory has a shorter life-span than IgG memory, and it may be that by the time of DIP-CGG challenge very few IgM memory cells remained. In support of this Devoino et al (1975) have shown that IgM memory to BSA is good at 2-5 weeks but cannot be detected after 8 weeks. Schlegel et al (1974) showed that anti-S treatment does not affect IgM memory responses to NIP, and Black and Inchley (1974) showed that IgM memory develops within the first 24 hours of priming, before primed T-cells are detected. It is thus unlikely that IgM memory merely reflects T-cell memory but leaves open the explanation for the $2^{\circ}$ type IgM anti-DNP response following carrier priming.
Carrier pre-immunisation has been shown to preferentially enhance the IgG response and bias it in favour of high affinity antibody (Cheers & Miller 1972; Kishimoto & Ishizaka 1973; Hurme, et al 1973). No such enhancement was seen in any of the experiments reported here. It may be argued that primed T-cells can only select and augment already responding IgG B-cells rather than recruit cells into the response, since there were no IgG PFC in the 1° responses to DNP-BSA and DNP-HSA or in the popliteal 1° response to DNP₀.₂ CRBC. However in the brachial anti-DNP₀.₂ CRBC response a good 1° IgG response was usually detected at day 5 but this response was totally absent after carrier (CRBC) pre-immunisation (Figs. 2-10c). This has not been repeated to ensure that it was not an artefact, but carrier pre-immunisation 2 weeks before challenge for a 10 week 2° response to DNP₀.₂ CRBC has also been shown to suppress (unpublished results).

Suppression, rather than help, following carrier priming has been demonstrated by other workers (Okumura & Tada 1973; Elson & Tada, et al 1975; Taylor 1974; Feldmann 1974; Arrenbrecht & Mitchell 1975; Eardley & Sercarz 1976). Two explanations have been proposed for this effect, the first being an excess of help, and the second being the result of suppressor T-cells. Recently primed carrier cells have often been transferred with normal spleen cells, either into culture or to X-irradiated recipients, for challenge with the hapten-carrier conjugate. Feldmann (1974a and b) has shown that low numbers of KLH primed T-cells can help in an in vitro DNP-KLH response whilst high numbers suppress. He has also shown that macrophages can abrogate this suppression when added to the culture and suggests that, in excess, the helper factor IgT may
react directly with specific lymphocytes to cause suppression instead of via macrophages for help. Elson and Taylor (1974) suggested that T-cells progress through a helper phase to a suppressor phase, and that the outcome of help or suppression depends on the relative stages of T and B cells in the response. Thus primed T-cells acting on virgin B-cells will suppress, but these same cells will enhance the response of memory B-cells. The results presented here showing suppression of the $1^\text{st}$ anti-DNP IgG response by CRBC priming, but enhancement of the $2^\text{nd}$ anti-DNP response by the same treatment (simultaneous priming with DNP-BSA and CRBC for challenge with DNP-CRBC) indicates that this may be so.

Other groups have demonstrated the presence of suppressor enable T-cells with distinct properties which/their separation from helper cells. Eardley and Scerarz (1976) showed that β-galactosidase (GZ) carrier-primed cells predominantly suppressed the TNP-GZ response for the first few days after immunisation but that help became manifest by day 9. Chan and Henry (1976) showed that following HRBC priming both helpers and suppressors for an anti-Lac (p-azophenyl-B-D-lactoside) response to Lac-HRBC co-exist at 3 days. The removal of suppressors by X-irradiation revealed a 9-fold increase in help. Okumura and Tada (1973) found that HRBC primed cells, generated by two injections at two weekly intervals, when transferred to normal recipients for challenge with DNP-HRBC not only caused suppression of an anti-DNP IgM response but also suppressed the anti-HRBC response. This may be the result of high levels of helper activity stimulating induction of suppressor cells in the normal recipients rather than of transfer of suppressor T-cells.
There is some evidence that anti-carrier antibody causes feedback inhibition of the anti-hapten response (Fidler et al 1972) by prevention of T-B cell interactions (Takatsu et al 1974). However high doses of passively administered anti-carrier antibody (anti-CGG) only suppressed the anti-hapten response to NIP-CGG by about 50% (Haughton & Makela 1973). Anti-carrier antibody could be acting in the experiments described here but probably very little is generated in response to a low dose of $10^6$ CRBC and even less will still be present by the time of challenge 5 weeks later. Since residual antibody would be predominantly IgG by that time, it might also be expected to preferentially suppress the IgM response. Finally, Fidler et al (1972) warn of artefacts in the help and suppression systems since they found that prior injection of $10^8$ SRBC suppressed an in vivo response to TNP-SRBC but the same injection enhanced an in vitro response.
CHAPTER 3

THE GENERATION OF MEMORY TO RBC AND DNP ANTIGENS
INTRODUCTION

Immunological memory develops early after priming and may continue to increase for many weeks or months. Both the kinetics of early memory generation and the subsequent maintenance, or increase, in the size of the memory cell pool are probably determined by the nature of the antigen under study. Few comparative studies between antigens have been made by any one group of workers thus, it is difficult to generalise concerning properties affecting memory generation. This lack of information was the starting point of work described in this chapter.

Memory to RBC antigens

It is difficult to study the early generation of memory in vivo. This is due both to the masking effects of an ongoing primary response and to regulatory events such as that of feedback control by antibody. Early workers overcame this problem by priming with a low dose of antigen so as to give only a poor primary response (Sercarz & Byers 1967). They showed that IgM memory developed within one day of priming to $2 \times 10^6$ SRBC although it continued to increase for a few days, the peak coinciding with that of the primary response. Memory for an IgG response to $2 \times 10^6$ SRBC was first seen on the sixth day after priming. However, following a priming injection of $10^8$ SRBC only a poor secondary response could be detected in vivo for at least 6 weeks (Safford & Tokuda 1971). This was probably due to feedback control exerted by circulating antibody since a population of memory cells had been generated and a secondary response could be expressed in vitro.

In vitro and in vivo adoptive transfers have frequently been used to release memory cells from the regulatory control of the primed host and thereby accurately assess the size of the memory
Cunningham (1969a and b) showed that IgM memory was fully developed within four days of priming with $2 \times 10^6$ SRBC iv and was maintained at the same level for 4-5 months. These experiments were later extended to distinguish between the separate kinetics of helper and precursor memory cell development, by using cross-reacting RBC in a hapten-carrier type system (Cunningham & Sercarz 1971). Precursor memory was only detected after high doses of cow RBC ($2 \times 10^7 - 2 \times 10^9$), memory for IgM being seen by day 4, and that for IgG by day 6 after priming. Both increased for at least two months after priming although IgM memory dropped between days 9-16 before rising again.

Cunningham and Sercarz (1971) suggested that IgM memory to low antigen doses (Sercarz & Byers 1967; Cunningham 1969a and b) may be a reflection of T-cell memory only. It is true that T-cell memory develops early in response to low doses of antigen and in the absence of significant antibody formation (Falkoff & Kettman 1972; Kappler & Hoffman 1973); also that primed T-cells can augment the primary IgM response (Falkoff & Kettman 1972; Cheers & Miller 1972; Fidler et al 1972) and that in some situations late IgM memory is mainly T-cell memory (Seppala et al 1976). However, other experiments have shown immediate development of IgM memory prior to development of memory T-cells (Sercarz & Byers 1967; Dutton & Mishell 1967; Black & Inchley 1974), and that IgM memory to DNP is unimpaired by removal of T-cells prior to challenge (Schlegel 1974). Black and Inchley (1974) developed a system for studying memory generation in vivo. They suppressed the primary anti-SRBC response with passive antibody and cyclophosphamide, at various intervals after priming, then studied the characteristics of the 12 week secondary response. This gave a
measure of the memory generated in the interval between priming and suppression. IgM and IgG memory developed after both high \((5 \times 10^8)\) and low \((5 \times 10^5)\) priming doses, IgM memory developing immediately upon contact with antigen while IgG memory was first detected 4 days after priming and peaked at day 7. It is uncertain why other workers (Cunningham & Sercarz 1971; Neiderhuber & Müller 1974) could not detect B-cell memory after low dose priming.

There are some contradictions concerning the late memory response to RBC antigens. Black and Inchley (1974) concluded that IgG memory reached a peak level within 7 days of priming and remained constant at that level for the next 11 weeks. In contrast, IgM memory dropped after the first week to reach primary levels by day 15. On the other hand, Cunningham and Sercarz (1971) showed that although some IgG memory developed within 6 days of priming, the memory population continued to increase in size for the next 2-3 months. They also noted a late rise in IgM memory after the initial drop between days 9-16.

There is some evidence that, following high priming doses of RBC, memory cells may immediately become recruited into the primary response, at the expense of the memory cell pool (Byers & Sercarz 1968; Hanna et al 1969). An in vivo transfer system was used to show that IgM memory was good 7 days after priming with \(2 \times 10^8\) SRBC but none could be detected by 2-3 weeks (Hanna et al 1969). Passive antibody treatment 1-4 days after priming resulted in a good 2-3 week adoptive IgM memory response, presumably by prevention of recruitment of these cells into the primary response. Black and Inchley (1974) compared the IgM memory response after priming with a high dose of SRBC \((5 \times 10^8)\) which invoked a strong primary response, with that after a low dose
(5 x 10^5) which did not induce a primary response. IgM memory was high within the first 24 hours of priming with either dose and was maintained at that level for the first week following the low dose. However, after high dose priming the initial high IgM memory pool became depleted in size for 1-2 days before regaining the same high level at day 4. They attributed this fall to the incorporation of IgM memory cells into the primary response. It should be noted, however, that in this system the IgM memory pool does not become permanently depleted.

It has been suggested that the fall in IgM memory between the first and second week of priming (Cunningham & Sercarz 1971; Black & Inchley 1974) might be due either to memory cell recruitment into the primary response or to conversion of IgM memory cells to IgG memory cells. The observations that recruitment of memory cells into the primary response only lasts for 2-3 days after priming, and that the IgG memory cell pool is complete within 7 days of priming (Black & Inchley 1974) invalidate both of these possibilities. An alternative explanation is that IgM memory cells are relatively short-lived and this fall between days 9-16 represents memory cell death. The longevity of IgM memory observed by others (Cunningham 1969) may indeed be a reflection of T-cell memory though this does not always seem to be true (Cunningham & Sercarz 1971).

Helper cell memory develops within 3-6 days after a wide range of RBC priming doses (2 x 10^5 - 2 x 10^9) (Cunningham & Sercarz 1971; Black & Inchley 1974). Cunningham and Sercarz (1971) showed that although peak "helper" memory was a little higher after a low than after a high antigen dose, it did not persist for as long and was falling by 16-30 days after priming.
That to a high antigen dose, increased between days 16-30. Persistent antigen might thus be important in the continued generation of T-helper memory but these estimates of T-memory lifespan are low compared with others (Sprent & Miller 1972). Black and Inchley (1974) found a good 12 week secondary response following both low and high priming doses of SRBC, presumably indicating the presence of primed T-cells, as well as B-cells, at that time.

In summary, IgM memory to RBC antigens develops immediately after immunisation but there are controversies concerning priming antigen dose requirements and lifespan of the memory cell pool. IgG memory is usually detected between days 4-7 after priming though again there are contradictions concerning dose requirements and time of peak memory response. There is general agreement that T-cell memory develops within 3-4 days of priming to a wide range of antigen doses but the importance of persistent antigen in maintenance of helper memory is also controversial.

Memory to protein antigens

The generation of memory to a wide range of non-RBC antigens, including a variety of proteins, has been studied. Priming with protein antigens generally gives an increase in secondary responsiveness over a period of several weeks. IgM memory is usually only seen early after priming and is particularly favoured by low antigen doses (Shirmacher & Rajewsky 1970; Folds & Stavitsky 1971). However, a late rise in IgM memory to NIP-MGG has been reported (Schlegel 1974).

IgG memory is usually seen within a week of priming with protein antigens (Fecsik et al 1964; Jacobson & Thorbecke 1969; Stavitsky & Folds 1972) but the degree of priming is low at this
point compared with highest observed levels. For instance, memory to KLH has been shown to increase for at least the first month in mice (Cerottini & Trnka 1970) and rabbits (Folds & Stavitsky 1971; Statitsky & Folds 1972). The study in mice was not continued beyond one month, but that in rabbits showed a plateau after the initial rise, then a further late rise for several months. This was also typical of memory generation to DT in rabbits. However, studies in mice indicated that the memory cell pool to both DT (Fecsik et al 1964) and TNP-KLH (Bullock & Rittenberg 1970) gained a maximum size within 6 weeks of priming. That to DT remained constant at peak levels for several months, but that to TNP fell gradually, although during the fall anti-TNP memory cells increased in avidity. Mohr and Krawinkel (1976) also demonstrated a fall in memory to HSA soon after it had attained peak levels. In an adoptive transfer system they showed that memory to HSA reached an early peak at 16 days, but very little remained at day 30.

Clonal memory development

Memory development within individual B-cell clones has been studied for the haptens NIP and DNP, using the isoelectric-focusing (IEF) assay for identification of specific clonotypes. Most systems have involved propagation of an individual clone through several transfer generations (Askonas, Williamson & Wright 1970; Askonas & Williamson 1972; Askonas et al 1972). Following antigen challenge in each new recipient, AFC (antibody forming cells) and new memory cells are formed, the measure of memory generation being the ability of spleen cells to transfer the response to the next recipient generation. Although such a system
is not directly analogous to memory generation from unprimed precursor cells, memory cells do develop with similar kinetics. No memory cells were detected 4 days after priming, but the response was good within 7 days and did not vary over the next 26 days (McMichael & Williamson 1974). This would indicate that the rise in memory seen for several months after priming with many protein and RBC antigens may reflect the complexity of the clonal response to these antigens, new clones gradually being recruited into the memory cell pool in response to persistent antigen. Confirmation of this comes from experiments of Kreth & Williamson (1973). They primed mice with NIP_{4.5} BGG, then transferred limiting numbers of spleen cells to a large number of irradiated recipients such that each recipient expressed only 0-3 clones upon challenge. Memory in this system was first detected 9 days after priming, increased to 30 days then remained constant to 100 days as judged by the gradual reduction in the number of cells required to transfer a monoclonal response. The number of unique clones in the memory cell pool increased for 72 days after priming and were eventually of higher affinity than those expressed early on. Despite the more limited clonal response 17 days after priming, some individual memory clones were more expanded at this time, as was shown by the detection of the same clone in several recipients.

Factors affecting priming

a) T-cell priming

T-cells can be primed by a wide range ($10^3 - 10^9$) of RBC doses (Cunningham & Sercarz 1971). Low doses ($10^3 - 10^5$) are most commonly used since these do not stimulate primary antibody formation (Falkoff & Kettman 1972; Dennert & Tucker 1972; Black & Inchley
1974). Non-immunogenic doses of DT, OVA and BoA (bacterial α-amylase) also prime well for a secondary delayed hypersensitivity or helper response (Salvin & Smith 1960; Takatsu et al 1974).

The T-cell memory pool is generated rapidly, sometimes being complete within 2 days of priming (Dutton & Mishell 1967; Greaves et al 1970; Romano et al 1975) or at least by 3-5 days (Shirrmacher & Rajewsky 1970; Cunningham & Sercarz 1971; Falkoff & Kettman 1972; Black & Inchley 1974; Mohr & Krawinkel 1976).

T-cell priming can occur in the presence of passive antibody (Safford & Tokuda 1971; Takatsu et al 1974) and may not require macrophages (Dennert & Tucker 1972; Kagnoff 1975) despite the fact that T-cell activation is totally dependent on macrophage contact (Oppenheim & Rosenstreich 1976). Dennert & Tucker (1972) fixed SRBC and CRBC with formaldehyde to prevent their processing by macrophages, and showed that in high numbers these cells primed T-cells very efficiently, although they could not induce a primary response. Kagnoff (1975) approached the problem in a different way by using Peyer's patch cells which contain few, if any, macrophages. He showed that SRBC "feeding" induced an excellent primed T-cell population in the Peyer's patches.

b) B-cell priming

It is generally agreed that B-cell priming is affected by three important factors - antigen dose, the time interval between priming and challenge, and the influence of accessory cells. The first two have already been considered in the individual sections on memory to RBC and proteins.

IgG memory to RBC antigens can often be detected as early as 4-5 days after priming, while that to proteins is seldom detected.
before the seventh or eighth day. It has been suggested that this difference may reflect differences in macrophage handling of these two types of antigen (Mohr & Krawinkel 1976). There is little information concerning the importance of macrophages for B-memory cell generation although the work of Dennert & Tucker (1972) indicated that they may be important. Formaldehyde treated RBC could not be solubilised by macrophage factors to stimulate B-cells in the normal way and in addition, no B-memory was generated by these cells. It has also been shown that RES (reticuloendothelial system) blockade by carbon injected 1-3 days prior to SRBC priming, delays and reduces the 4 week secondary IgG response but has no effect on the secondary IgM response (Sabet & Friedman 1969).

Macrophages are essential for B-cell activation in response to both RBC and protein antigens although the mechanism by which they work differs in each case (Oppenheim & Rosenstreich 1976). B-cell activation in response to a protein antigen requires direct contact between B-cell and macrophage plus an additional specific signal from the activated T-cell. It is suggested that the T-cell produces a factor, perhaps monomeric IgM, which complexes with antigen on the macrophage surface. B-cells interact with this complex and become activated (Feldmann et al 1973). Other models are essentially similar to this, although the nature of the T-cell factor is somewhat controversial.

Direct macrophage-B-cell contact is not required in the response to SRBC. Instead, the macrophage releases a factor which solubilises the RBC, thereby rendering them T-cell and macrophage independent (Oppenheim & Rosenstreich 1976). Other workers have confirmed that the immune response to solubilised SRBC stroma is
macrophage independent (Feldmann & Palmer 1971). Since RBC antigens are T-dependent, the T-cell must be acting at some point, perhaps by activating the macrophage or producing non-specific factor (TNF) to augment the B-cell response (Oppenheim & Rosenstreich 1976). However, another pathway has also been proposed, similar to that for proteins, involving macrophage-B-cell contact plus T-cells.

Antigen handling by macrophages is clearly important for response to proteins and may be important for at least one mechanism by which responses to RBC are induced. Efficiency of handling varies for different antigens and even for different forms of the same antigen. Nakano (1976) compared the antigen trapping and degradation of soluble BSA, heat aggregated BSA and alum precipitated BSA. These antigens increase in immunogenicity in the order shown. Soluble BSA was poorly trapped by macrophages, correlating with its poor immunogenicity since macrophages containing soluble BSA were immunogenic. However heat aggregated BSA was extremely efficiently trapped, more so than alum BSA despite the greater immunogenicity of the latter. This was probably due to the much higher retention of alum BSA within the macrophage. Retained material is either on the macrophage surface or internalised and slowly released from the cell (Oppenheim & Rosenstreich 1976).

There are some indications that primed T-cell help may be a prerequisite for B-cell priming, particularly for IgG memory. This would be compatible with the requirements for T-cell help in expression of both primary and secondary IgG responses (Raff 1970; Roelants & Askonas 1972). B-cell memory is first seen 24 hours after T-cell priming is complete and thus could well involve T-cell help (Black & Inchley 1974), although addition of primed T-cells at
the time of priming does not induce earlier B-cell memory. Passive anti-BαA (bacterial α-amylase) antibody has been shown to prevent expression of both primary and secondary responses to BαA by interfering with cell-co-operation, and at the same time to prevent B-memory generation (Takatsu et al 1974). Anti-SRBC antibody, however, does not prevent memory generation although it does prevent T-B co-operation for memory expression (Safford & Tokuda 1971). Memory to the T-independent antigens, DNP-SIII and DNP-Levan, is usually absent or consists only of low levels of IgM memory (del Guercio et al 1974; Lerman et al 1975). However, the T-independent antigen, Brucella abortus (BA), stimulates good IgM and IgG memory (Mond et al 1974). The significance of this observation is uncertain since T-cells are efficiently stimulated by BA, despite the fact that they are not required for expression of primary and secondary responses. Memory develops in response to the heavily dinitrophenylated conjugate DNP₄₂BSA, which has many characteristics of a T-independent antigen, but can only be expressed after challenge with a low epitope density conjugate (Vachek & Kolsh 1975).

Two important approaches to this problem have involved the use of adult thymectomised, irradiated and bone-marrow reconstituted (ATxBM) mice and nu/nu mice. The former, when primed with MSH or TT (Roelants & Askonas 1972) showed good memory provided that T-cells were added at the time of challenge. Nu/nu mice primed with FGG, DNP-BSA or SRBC also developed good memory, but again it was only expressed upon addition of primed T-cells (Schrader 1975; Diamenstein & Blitstein-Willinger 1974). However, Schlegel (1974) has shown that nude mice do require T-cell help for both generation and expression of B-cell memory to NIP-HGG. The problem with both of these approaches is that where priming was effective, there may have been sufficient
T-cells to generate 'help'. Schrader (1975) demonstrated the presence of T-cells in ATxBM mice but could not detect them in the nu/nu mice. Okumura et al (1976) overcame this problem by using allotype suppressed mice which will give a near normal IgG response, deficient only in the Ig-Ib allotype due to the lack of specific helper cells for that allotype. They studied Ig-Ib memory development and compared it to the rest of the IgG memory, developing normally in the presence of T-cells. The main conclusion from these studies was that a pool of B-memory cells developed independently of T-cells, the affinity of the memory cells being dependent on the priming antigen dose. However, increase in the affinity of memory cells in response to a booster injection of antigen was totally dependent upon T-cell help.

**Maintenance and increase in memory**

Maintenance of the overall size of the memory cell pool for several weeks, or months, after priming may be related solely to the long lifespan of memory lymphocytes. However it could also result either from recruitment of newly produced virgin cells or the restimulation of existing memory cells in response to persistent antigen*. It is well established that memory populations divide to produce further memory cells after fresh antigenic challenge (Askonas & Williamson 1972; McMichael & Williamson 1974; Nakashima & Kato 1975; Feldbush & van der Hoven 1976) and persistent antigen could have the same effect. Increase in memory over several weeks, or months, after priming may reflect either an expansion of existing clones, recruitment of new memory clones, or both. There is evidence from experiments of Kreth & Williamson (1974) that clonal recruitment occurs for at least 72 days after priming to NIP.

*For discussion of work on persistent antigen see Chapter 6.*
The functional half-life of memory cells in the absence of antigen has been estimated by transfer of primed cells to X-irradiated recipients, leaving different intervals between transfer and challenge (Celada 1967 & 1971; Feldbush 1973; McGregor & Mackaness 1975).

Memory decay was biphasic indicating the existence of two populations of memory cells, one short-lived with $t_1 = 4-14$ days, and the other long-lived with $t_2 = 40-126$ days. More recent experiments have indicated that the short-lived population may be an artefact of the transfer system used (Feldbush et al 1974). Estimates of the half-life of the long-lived memory population to DNP-BGG in the spleen varied between 40-126 days, while the half-life for those in thoracic duct and lymph nodes was approximately 100 days (Feldbush et al 1974). However, transfer of spleen cells to normal recipients for residence, before a second transfer and assay in X-irradiated hosts indicated that memory cells have a half-life of only 30 days. These experiments did not distinguish between B and T cells but Elson and Taylor (1976) used a similar double transfer to measure the functional half-life of B-virgin and B-memory lymph node cells specific for TNP. They found the half-life for virgin cells was about 7 days and that for memory cells 14-21 days.

The importance of persistence of the priming antigen in maintenance of immunological memory will remain uncertain until these discrepancies in estimates of memory cell half-life can be resolved. Strober (1976) has recently reported the existence of a short-lived memory blast cell in the thoracic duct lymph, the generation of which is dependent on persistent antigen. These blast cells can be detected for several weeks after priming to DNP-BSA but do not persist for several months as do memory small lymphocytes.

Aim of study
An attempt was made to resolve the differences between the results of Cunningham and Sercarz (1971) and Black and Inchley (1974) concerning a late rise in memory to RBC antigens. The former workers immunised CBA mice with cow RBC intraperitoneally (ip) and used an adoptive transfer system to assess the extent of memory development in the spleen before transfer. The latter workers also immunised CBA mice ip, but in this case with SRBC. They halted the primary response at intervals after priming then challenged the mice at 12 weeks to assess the extent of memory development before the primary response was halted. The present experiments were designed using the same source of animals and materials as Black and Inchley (1974) but the adoptive transfer system of Cunningham and Sercarz (1971) to determine whether differences in their results could be attributed to the different techniques used, or to some other factor.

One difference between their experiments and those in the present work, has been the use of subcutaneous immunisation and assay of the brachial lymph node response instead of ip immunisation for assay of the spleen. In addition to assay of the secondary PFC response, the clonal heterogeneity of memory cells to SRBC has been studied using the IEF assay, to see if the memory pool is heterogeneous as early as 7 days after priming, when IgG memory is high as judged by the PFC assay. If a late rise in memory were detected, such a system should determine whether this rise was due to expansion of existing memory clones or to recruitment of new clones.

The kinetics of memory development to DNP have been studied using CRBC and BGG as carrier molecules, with the intention of determining the role of the carrier substance in controlling the characteristics of priming for a given hapten. Such a comparison has not previously been made, and it was hoped that the study would
resolve some of the conflicting reports on memory to cellular and soluble antigens.

**MATERIALS AND METHODS**

1. Animals, antigens, immunisation and PFC assay were as described in Chapter 2.

2. **Haemagglutination (HA) assay**
   Mice were bled from the retro-orbital sinus. Serial dilutions of serum were made in BIOZZI PBS (appendix) plus 0.1% gelatin, in microtitre trays (Linbro Scientific Co. Inc., Hamden, Conn.). 1% RBC was added and the plates incubated at 37°C for 1 hour, then left overnight in the cold before reading. 2-mercaptoethanol (2ME) (BDH Chemicals Co.) was added for detection of 2ME resistant (2MER) titres.

3. **Isoelectricfocusing (IEF) assay**
   These assays were performed on flat plate polyacrylamide gels. Antibodies to SRBC were detected by bands of lysis of SRBC incorporated in an agarose overlay, as first described by Phillips and Dresser (1973a). Subbed glass plates (Ilford Research Labs.) were baked overnight before use. A 5.13% acrylamide solution containing acrylamide and NN-methylenesbis acrylamide (appendix) was polymerised by addition of a 10% Analar ammonium persulphate solution using a 10% TEMED solution (NNN' tetramethylethylene diamine) as catalyst. Ampholine carrier ampholytes, pH 5-8 (LKB Ltd.) were included in the gel. For details see appendix.

   1.0M H₃PO₄ and 1.0M NaOH were initially used as electrode solutions applied to the gel on electrode strips (LKB). The concentration of these buffers was later reduced to 0.5M in an attempt to eliminate pH drift towards the cathode. The gels were pre-run at constant current, 5 m amps, until the voltage reached 150, then switched to
constant voltage. 10µl samples of undiluted serum were applied to the gels on cellulose acetate paper strips, 4.0 x 7.0 mm. The sample papers were either removed after 1 hour or left overnight. The gels were run at 150 volts for 18-20 hours, then the electrode strips were removed and gels washed for 30 minutes in 3 changes of HBSS (Oxoid, England). They were heated gently on a level, warmed plate and a solution of 25% RBC in 0.75% agarose at 49°C was pipetted over the surface of each gel. A rabbit anti-mouse light chain developing serum was included in this overlay. After the agarose had set, the gels were placed in a moist box at room temperature for 2 hours, then incubated at 37°C with a 1:10 solution of guinea-pig complement until bands appeared. Bands of lysis were photographed using contact prints on Kodak Bromide No. 4 paper. Unless otherwise stated, samples within a single experiment were run on the same gel.

4. Adoptive transfers

In an in vivo adoptive transfer system there is a linear relationship between the number of cells transferred to the recipient and the magnitude of a response given by the recipient (Celada 1967; Bosma et al 1968; Cunningham 1969a and b). At a dose of 10⁷ spleen cells this has been shown to be true for both primed and virgin cells (Bosma et al 1968).

a) Preparation of cell suspensions

Brachial and axillary lymph nodes were gently dissociated using a ground glass homogeniser, seived through a stainless steel screen and washed twice in HBSS. Viability was assessed using the trypan blue dye exclusion test. Either 10⁷ or 2 x 10⁷ viable lymph node cells, pooled from 5 donor mice, were injected intravenously (iv) into a tail vein of X-irradiated recipients. Recipient mice were immediately challenged with 10⁸ RBC iv.
b) **Irradiation**

Irradiations were mainly performed using a Westinghouse therapeutic machine. Mice were placed in a perforated polystyrene box and exposed to 900r or 750r (200 kv, 15 ma, filtration 0.5 mm copper, 1 mm aluminium, focus to target distance 75 cm, dose rate 66 rads per minute with back and side scatter).

In some of the later experiments mice received $\gamma$-irradiation from a $^{137}$Cs source at a dose rate of 39.28 rads per minute $\pm 3.6\%$. 
RESULTS AND DISCUSSION

1. The kinetics of the adoptive primary (1°) and secondary (2°) responses to SRBC

The kinetics of the adoptive PFC response of recipients of unprimed cells, or of day 7 or 21 SRBC-primed cells, are shown in Fig. 3.1. Peak responses were delayed compared with those in intact animals, the peak 1° IgM being at day 6 and peak 2° IgM at day 5. A more prolonged 2° IgM response was seen in recipients of 21 day than of 7 day primed cells. 2° IgG PFC were detected earlier after cell transfer than were 1° PFC (day 4 compared with day 6) and rose to a plateau by day 6. Lymph node cells primed 21 days before transfer gave a higher IgG response than 7 day primed cells, on all days assayed, statistically significant on days 5, 6 and 7 (p < 0.01).

Recipient mice were also bled and the IEF spectra for recipients of primed cells can be seen in Fig. 3.2. No bands were seen in day 4 sera and only a few in day 5, despite the good IgG PFC response. However by day 6, both groups showed intense and heterogeneous IEF spectra, only marginally improved towards day 7. For the 3 week primed cells this improvement showed as an increase in the intensity of individual bands (i.e. clone size) rather than an increase in the number of bands (new recruitment). The same conclusion could not be drawn for the day 7 primed cells because individual bands were hard to distinguish. It seems likely, from these and other experiments, that there was approximately a 24 hour delay between detection of IgG PFC and detection of serum antibodies by the electrofocusing technique. Although the number of bands per clone (spectro-type) is variable, it is generally held that the average number is 3-4 (Awdeh et al 1970; Phillips & Dresser 1975). Phillips and Dresser (1975) have compared peak PFC
Fig. 3.1 Kinetics of the adoptive $1^0$ and $2^0$ response to SRBC

PFC response in groups of 5 irradiated recipients (900r) of $10^7$
poled brachial and axillary lymph node cells and $10^8$ SRBCiv.
Donors were primed with $5 \times 10^6$ SRBC sc in the front footpads
21 days $\rightarrow$, or 7 days $\bullet\bullet\bullet$ before transfer, or
were unprimed $\rightarrow$. 
Fig. 3.2  Kinetics of the adoptive \(2^0\) response to SRBC.

IEF spectra of sera from irradiated (900r) recipients of \(10^7\) primed lymph node cells and \(10^8\) SRBCiv. Donors were primed with \(5 \times 10^6\) SRBCsc (a)7 or (b)21 days before transfer. Recipients were bled on days 4(A and E), 5(B and F), 6(C and G) and 7(D and H) after transfer.
responses with the number of spectrotypes seen after IEF, and have estimated that there are about $7 \times 10^3$ anti-SRBC PFC per clone. Similar estimates were difficult to make from the work presented here, since bands were hard to count accurately and smears were often seen between bands. The day 7 sera of recipients of 21 day primed cells contained an average of 20 bands. On the assumption that this represents about six major clones, a figure of approximately $4 \times 10^3$ PFC per clone is derived. This compares well with that of Phillips and Dresser (1975) and with other estimates of anti-SRBC clone size ranging from a background of 40 to 28,000 PFC per clone (review Cunningham 1973).

In the following experiments, unless otherwise stated, a day 6 assay was chosen since this detected both peak IgG PFC and a fully developed clonal spectrum at a time when primary IgG responses were still low. Although IEF spectra were more intense at day 7, the primary response was rising by then and might have confused interpretation of the results, particularly for the PFC assay.

2. Rate of priming to SRBC

The kinetics of early priming to low doses of SRBC were studied by priming donor mice in the two front footpads with $5 \times 10^5$ or $5 \times 10^6$ SRBC sc 2, 3, 4, 7, 14 and 21 days before adoptive transfer of pooled brachial and axillary lymph node cells. In some experiments B. pertussis was given with priming injections. Recipient mice were bled and their spleens removed for a PFC assay 6 days after transfer.

The PFC assay

IgM memory proved variable, and sometimes no increase over adoptive primary levels was detected (Fig. 3.3b). In the
Fig. 3.3 Kinetics of priming to SRBC.

Two experiments showing day 6 splenic PFC responses in groups of 5 irradiated (900r) recipients of (a) 2x10^7 or (b) 10^7 pooled brachial and axillary lymph node cells and 10^8 SRBCiv. Donors were immunised with 5x10^5 SRBCsc. Primary IgM and IgG responses ± 1 se are also given. ○—-○, IgG PFC; ■---■, IgM PFC.

2* 1 mouse died before assay.
7* 4 mice died before assay.
Interval between priming and transfer (days)

mean log_{10} PFC ± se

1° IgM

1° IgG

2°
Fig. 3.4 The effect of B.pertussis on priming to SRBC.

Day 6 splenic PFC response in groups of 5 irradiated (900r) recipients of $10^7$ pooled bronchial and axillary lymph node cells and $10^8$ SRBCiv. Donors were immunised with $5 \times 10^6$ SRBCsc + $5 \times 10^3$ B.pertussis. Primary IgG and IgM responses ± 1 se are given.

- - - IgG PFC; ■ ■ ■ IgM PFC.
Days between priming and transfer

mean log$_{10}$ PFC ± se

2 3 4 5

Days between priming and transfer

log$_{10}$

log$_{10}$
experiment shown in Fig. 3.3a there was an early rise between 2 and 3 days after priming in the capacity to give IgM PFC but this may not represent IgM priming since these levels were not significantly higher than a primary response. However it should be remembered that a PFC assay 6 days after transfer detects peak 1° IgM responses but the 2° IgM may be falling at this time (Fig. 3.1). In cells primed for more than four days before transfer, the adoptive 2° IgM PFC response was sometimes similar (or slightly higher), to that of 4 day primed cells (Fig. 3.3a and Fig. 3.4), and sometimes lower (Fig. 3.3b).

IgG memory had developed in donor mice primed with $5 \times 10^5$ SRBC sc (Fig. 3.3a and b), $5 \times 10^6$ SRBC sc (Fig. 3.1) or $5 \times 10^6$ SRBC + $5 \times 10^8$ B. pertussis sc (Fig. 3.4), 7 days before transfer. It was often also evident in 4 day primed cells (Figs. 3.3b and 3.4) but there was sometimes considerable variability between responses of recipients of these recently primed cells (Fig. 3.3a). It is possible that day 4 is a transitional time in memory development and that the transfer of limiting numbers of primed cells produced this variability, although $2 \times 10^7$ rather than $10^7$ cells were transferred in the experiment with the greatest variability (Fig. 3.3a). Adoptive 2° responses given by cells primed for more than 7 days before transfer were often similar to those of day 7 primed cells, particularly after priming with $5 \times 10^5$ SRBC sc. However, after priming with $5 \times 10^6$ SRBC sc the memory cell pool sometimes continued to increase in size for 1-2 weeks (Figs. 3.1 and 3.4).

The IEF assay

A broad spectrum of clones was represented in the memory cell pool within 7 days of priming with $5 \times 10^5$ SRBC sc, as evidenced
by the heterogeneous IEF spectra of recipients of 7 day primed cells (Fig. 3.5). Few bands were detected in recipients of unprimed cells, or 2 or 3 day primed cells. The responses of 4 day primed cells were variable between experiments, memory sometimes being pronounced at this time (Fig. 3.5b). However even where pronounced, clonal heterogeneity increased after day 4 of priming, there being more bands in recipients of cells primed 14 days before transfer. In the experiment shown in Fig. 3.6 the memory cell pool was highly heterogeneous with respect to clones within four days of priming, and clonal heterogeneity did not increase after this time. This may have been the consequence of priming with a higher antigen dose ($5 \times 10^6$ SRBC sc), or of priming with $B. pertussis$ which might increase the rate of proliferation of memory precursors (Chapter 5).

Cells transferred later after priming (21 days) sometimes gave weaker spectra than more recently primed cells (Fig. 3.6). This observation was reflected in the haemagglutination (HA) titres (Table 1) but did not correlate with the PFC responses (Fig. 3.4) which were greatest after transfer of day 14 primed cells. However, in another experiment in which mice were similarly primed with $5 \times 10^6$ SRBC + $5 \times 10^8$ $B. pertussis$ sc, the spectra given by cells primed 21 days before transfer were more intense than those of 7 day primed cells (Fig. 3.7a). This does not correlate with either the PFC responses or the HA titres (Table 2), both of which were similar for day 7 and 21 primed cells. It is possible that since developing serum was not used for detection of IgG antibodies in the HA assay, this assay was not sufficiently sensitive to detect differences between the groups. The experiment in Fig. 3.7b indicates that yet another difference sometimes exists between
**Fig. 3.5  Kinetics of priming to SRBCsc.**

IEF an lyses of sera of irradiated (900r) recipients of (a) $2 \times 10^7$ or (b) $10^7$ pooled brachial and axillary lymph node cells and $10^8$ SRBCiv. Recipient mice were bled 6 days after transfer and challenge. Donors were immunised with $5 \times 10^5$ SRBCsc 2(D), 3(A), 4(B and E) or 7(C and F) days before transfer.
Fig. 3.6 The effect of B. pertussis on priming to SRBCsc.

IEF analyses of sera of irradiated (900r) recipients of \(10^7\) pooled bronchial and axillary lymph node cells and \(10^8\) SRBCiv. Donors were either unimmunised (A) or immunised with \(5\times10^6\) SRBC + \(5\times10^8\) B. pertussis sc 4(B), 7(C), 14(D) or 21(E) days before transfer. Recipient mice were bled on day 6 after transfer.
**TABLE 1**

Adoptive 2° responses given by cells primed with 5 x 10^6 SRBC sc + 5 x 10^8 E. pertussis

<table>
<thead>
<tr>
<th>Interval between priming and transfer</th>
<th>Haemagglutination titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td>mean ± s.e.</td>
</tr>
<tr>
<td>21 days</td>
<td>7.0 ± 0.9</td>
</tr>
<tr>
<td>14 days</td>
<td>8.6 ± 0.2</td>
</tr>
<tr>
<td>7 days</td>
<td>8.2 ± 0.4</td>
</tr>
<tr>
<td>4 days</td>
<td>6.0 ± 0.6</td>
</tr>
<tr>
<td>Unprimed</td>
<td>0.8 ± 0.5</td>
</tr>
</tbody>
</table>

Haemagglutination titres of day 6 sera of irradiated (900r) recipients of 10^7 pooled lymph node cells and 10^8 SRBC iv. 2 MER titres of recipients of day 7 primed cells are significantly higher than those of day 4, 14 or 21 primed cells (p < 0.01).
Fig. 3.7 Comparison of 2\(^0\) responses given by cells primed 7 and 21 d ys before transfer.

IEF analyses of day 6 sera from irradiated (900r) recipients of 10\(^7\) pooled br chiel and axillary lymph node cells and 10\(^8\) SRBCiv.

Donor mice were immunised with (a)5x10\(^6\) SRBC + 5x10\(^8\) B. pertussis sc 7(A) or (b)5x10\(^5\) SRBCsc 7(C), 10(D) or 21(E) d ys before transfer.
TABLE 2

Comparison of 2° responses given by cells primed 7 or 21 days before transfer

<table>
<thead>
<tr>
<th>Day of priming</th>
<th>Mean log_{10} PFC ± s.e.</th>
<th>Haemagglutination titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM</td>
<td>IgG</td>
</tr>
<tr>
<td>7</td>
<td>3.42±0.22</td>
<td>4.34±0.13</td>
</tr>
<tr>
<td>21</td>
<td>2.31±0.17</td>
<td>4.29±0.21</td>
</tr>
</tbody>
</table>

Day 6 PFC responses and HA titres of day 6 sera of irradiated (900r) recipients of 10^7 lymph node cells and 10^8 SRBC iv.
cells primed for different lengths of time before transfer. In this case the $2^\circ$ response becomes more clonally restricted with time after priming. Since the adoptive $2^\circ$ IgG PFC responses were high in all groups this must indicate that some memory clones became preferentially expanded at the expense of others. There is no evidence for this from IEF in which restricted bands should have been more intense unless each AFC was producing less antibody.

It is clear that correlations between the PFC and IEF assays were good following transfer of recently primed cells (2-7 days) but were less reliable following transfer of cells primed for longer intervals of time. The possible explanations for this will be considered in more detail later.

3. Is there a late rise in memory to SRBC?

The experiments so far considered indicated that IgG memory, as measured in a PFC assay, reached peak levels within the first or second week of priming. Two experiments (Figs. 3.3a and 3.4) showed a slight, but not statistically significant, fall in IgG PFC levels after this peak. In the following experiments the time interval between priming and transfer was increased to see if this fall in PFC numbers could be repeated and, if so, to see whether at later times, memory fell to primary levels or rose again as shown by Cunningham and Sercarz (1971).

It was found that the capacity to generate IgG PFC remained constant for at least 5-7 weeks after priming with $5 \times 10^6$ SRBC sc (Fig. 3.8). The large variance within the recipients of 14 day primed cells was due to 2/5 mice poorly responding and probably relates to hazards of the transfer technique. The same 2 mice were clearly producing very little antibody as evidenced by their weak IEF spectra (Fig. 3.9a). However poor IEF spectra do not
Fig. 3.8 Duration of memory to SRBC

Day 6 splenic PFC response in groups of 5 irradiated (900r) recipients of $10^7$ pooled brachial and axillary lymph node cells and $10^8$ SRBCiv. Donor mice were primed with $5 \times 10^6$ SRBCsc. Primary IgM and IgG responses ± 1 se are also shown. Duplicate experiments

■...■, IgM PFC; □—□, IgG PFC.
Weeks between priming and transfer

### a)

#### $\log_{10}$ mean PFC ± se

- $1^{st}$ IgM
- $1^{st}$ IgG

### b)

#### $\log_{10}$ mean PFC ± se

- $1^{st}$ IgM
- $1^{st}$ IgG

---

**Notes:**
- $E = E_0$
Fig. 3.9 Duration of memory to SRBC

IEF analyses of sera from irradiated (900r) recipients of $10^7$
pooled brachial and axillary lymph node cells and $10^8$ SRBCiv.
Donors were primed with $5 \times 10^6$ SRBCsc 1(A and E), 2(B), 3(C), 4(D),
5(F) or 7(G) weeks before transfer. a, b: different experiments.
A, B, C and D were run on a single gel.
F and G were run on the same gel but E was run on a separate gel.
always correlate with low IgG PFC numbers, as is the case for the 14 other low antibody-producer in the day/primed group and the low producers in the 21 day primed group. A day 7 bleed after transfer (instead of day 6), would probably have detected circulating antibody in these mice. It is, nevertheless, clear from the IEF analyses shown in Fig. 3.9 that there was only a little, or no, deterioration in the intensity of the spectra produced by cells transferred 5-7 weeks after priming compared with antibody production by cells primed one week before transfer.

Since both PFC and IEF assays of these experiments indicated that memory remains relatively constant over the first 5-7 weeks after priming, it is probable that this truly represents the relative distribution of memory cells among committed clones. It follows that declines, when detected, were artefacts of the system which are as yet poorly understood. The $2^\circ$ IgM PFC response also remained relatively constant over this period of time although there was a slight fall from day 7 levels in one experiment before a plateau was attained (Fig. 3.8). This was confirmed by other experiments shown in Chapter 6 (Fig. 6.3). There is then no support either for a fall in IgM memory to $1^\circ$ levels by the end of the second week after priming (Cunningham & Sercarz 1971; Black & Inchley 1974) nor for the late rise in IgM memory over a period of several months (Cunningham & Sercarz 1971).

4. Rate of priming to DNP when conjugated to different carriers

In the following series of experiments, the relative importance of hapten and carrier determinants in governing the kinetics of priming to the hapten have been studied. This has been done by comparing priming to DNP after an sc injection of either DNP-CRBC or DNP-BCG. Since most studies on memory
development have utilised SRBC or cow RBC it was first necessary to compare the kinetics of anti-CRBC priming with those of anti-SRBC priming to ensure that it is possible to generalise about memory generation to RBC.

**Early priming to CRBC**

Mice were primed in the front footpads with $5 \times 10^7$ CRBC sc. This high dose was chosen since high doses of dinitrophenylated CRBC are best for inducing anti-DNP priming (Chapter 2). $10^7$ pooled brachial and axillary lymph node cells were adoptively transferred to irradiated recipients for challenge, and the $2^\circ$ PFC response was determined 6 or 7 days later. The results indicated that excellent IgG memory developed within 7 days of priming in one experiment (Fig. 3.10a) but none was seen until day 14 in the other (Fig. 3.10b), after which it remained constant for a week. IgM $2^\circ$ responses given by cells primed 7, 14 and 21 days before transfer were all high.

Isoelectric focusing of the anti-CRBC response has not proved very successful, smears of lysis often being observed instead of clear bands (Fig. 3.11). This assay might be improved by use of a different source of complement, or reduction in the priming antigen dose to restrict the number of clones recruited. However it was clear from these gels that although very little antibody was produced in the adoptive $1^\circ$ response, large amounts were produced by cells primed 7 days before transfer. Once again (see Fig. 3.6) the IEF spectra of day 14 and 21 primed cells was weaker than that of day 7 cells, in direct contradiction to the higher PFC numbers at these times (Fig. 3.11b). In fact, it is interesting that although 3/5 mice receiving 7 day primed cells had no IgG PFC, all 5 showed strong IEF spectra. It may be that peak $2^\circ$ IgG responses had fallen by day 7.

75.
Fig. 3.10  Kinetics of priming to CRBC.

Day 7(a) or 6(b) splenic PFC responses of irradiated (900r) recipients of $10^7$ pooled brachial and axillary lymph node cells and $10^8$ CRBCiv. Donors were immunised with $5 \times 10^7$ CRBC + $5 \times 10^8$ B. pertussis sc. 10

IgM and IgG PFC responses ± 1 se are given.

■■■■■, IgM PFC; □□□□□, IgG PFC.
Days between priming and transfer

a) IgG
   IgM

b) mean log_{10} PFC ± se

- 1^o IgM
- 1^o IgG

<table>
<thead>
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<th>Days</th>
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Fig. 3.11 Kinetics of priming to CRBC.

IEF spectra of day 6 sera from irradiated (900R) recipients of $10^7$ pooled bronchial and axillary lymph node cells and $10^8$ CRBCiv.
Donors were either unprimed (A) or primed with $5 \times 10^7$ CRBC + $5 \times 10^8$ E. pertussis sc 7(B and C), 14(D) or 21(E) days before transfer.
Fig. 3.12 Kinetics of carrier priming after immunisation with DNP-CRBC.

Day 7 anti-CRBC PFC responses of irradiated (900r) recipients of $10^7$ pooled brachial and axillary lymph node cells and $10^8$ DNP-CRBCiv. Donors were primed with $5 \times 10^7$ DNP-CRBC + $5 \times 10^8$ B.pertussis sc.

$1^0$ IgM and IgG responses $\pm$ 1 se are also given.

■——■, IgM PFC; □——□, IgG PFC.
Days between priming and transfer

mean log_{10} PFC ± se
Fig. 3.13 Kinetics of carrier priming after immunisation with DNP-CRBC.

IEF spectra of day 7 sera of irradiated recipients (a, 900r and b, 750r) of $10^7$ pooled brachial and axillary lymph node cells and $10^8$ DNP-CRBCiv. Donors were either unprimed (A and E) or primed with $5 \times 10^7$ DNP-CRBC + $5 \times 10^3$ B. pertussis 4(B), 7(C and F), 14(D) or 21(G) days before transfer.
It is clear that priming to CRBC followed similar kinetics to those for priming to SRBC, a high $2^\circ$ IgG PFC response being evident in recipients of cells primed 7-14 days before transfer, and memory remaining constant for the following week. The clonal spectra were highly heterogeneous, and most intense after transfer of recently primed cells. To ensure that DNP haptenation did not affect the kinetics of priming to CRBC, mice were primed with $2 \times 10^7$ heavily dinitrophenylated CRBC (DNP$_2^{0.2}$CRBC) with B. pertussis, and the adoptive $2^\circ$ responses to the carrier (CRBC) were assayed following transfers at intervals after priming. Again the capacity to give a $2^\circ$ IgG PFC response was clearly seen within 7 days of priming, peaking at day 14 then remaining constant for the following week (Fig. 3.12). IgM memory developed between days 4 and 7 after priming then remained roughly constant at day 7 levels. IEF assay of this experiment was poor, but that of similar experiments again indicated that bands were most intense in recipients of recently primed cells (days 4 and 7) (Fig. 3.13).

**Priming to DNP**

A preliminary experiment was conducted to compare the kinetics of priming to SRBC with those to DNP conjugated to either CRBC or BGG. B. pertussis was included in this and in all other DNP experiments, as well as in the SRBC controls, and was injected by the same route and at the same time as antigen. In this experiment the PFC responses of cells transferred 7 and 21 days after priming were assayed. As in previous experiments there was an excellent IgG PFC response to SRBC from both day 7 and 21 primed cells (Fig. 3.14). The high $1^\circ$ IgG response probably reflects both the use of a day 7 rather than a day 6 assay, and the use of 750r recipients. In contrast to the effects of anti-SRBC priming, day 7
anti-DNP₀.₂-CRBC primed cells did not give a 2° IgG PFC response, but memory had developed by 21 days after priming. IgM memory was seen on both days. It would thus seem that IgG memory to DNP developed later than that to the CRBC carrier to which it was conjugated (Fig. 3.12). However an excellent 2° IgG response to DNP was given by cells primed with DNP-BGG 7 days before transfer, and 21 day primed cells showed a further significant increase. There was thus good priming to DNP by DNP-BGG within 7 days but it continued to increase during the next two weeks.

Two more experiments were done to extend these observations. Mice were primed with DNP₀.₂-CRBC on days 4, 7, 14 and 21 before transfer. The PFC responses confirmed the preliminary results in that there was no IgG PFC response given by 7 day or 14 day primed cells but good memory had developed by day 21 (Fig. 3.15a and b). IgM memory rose to a peak within 7-14 days of priming, before falling slightly. 1° IgM levels were higher after a day 6 assay (Fig. 3.15b) which detects the peak response.

The preliminary experiment in which donor mice were primed with DNP-BGG was also extended to include transfers on days 4, 7, 14 and 21 after priming. The results are shown in Fig. 3.16. No IgG PFC were seen in recipients of 4 day primed cells but there was good memory by day 7, confirming the results of Fig. 3.14. However in this experiment the IgG memory dropped dramatically between day 7 and 14 to a level not above that of the primary. The reason for this remains unclear but it could have been due to an unsatisfactory transfer of cells. IgM memory was evident in recipients of day 4 and 7 primed cells but fell slightly for later transfers. This is in agreement with Fig. 3.14c where IgM memory also falls between days 7 and 21. It was not determined why a fall in IgG memory was
Fig. 3.14  Comparison of kinetics of priming to SRBC and DNP.

Day 7 splenic PFC responses in groups of 5 irradiated (750r) recipients of $10^7$ pooled brachial and axillary lymph node cells and (a) $10^8$ SRBCiv,
(b) $10^8$ DNP-CRBCiv or (c) 50μg DNP-BGGiv. Donors were primed with
(a) $5 \times 10^6$ SRBC + $5 \times 10^8$ B. pertussis sc, (b) $5 \times 10^7$ DNP-CRBC + $5 \times 10^8$
B. pertussis sc or (c) 50μg DNP-BGG + $5 \times 10^8$ B. pertussis sc. 10 IgM
and IgG responses ± 1 se are also given.

■ ■ ■, IgM PFC;      □——□, IgG PFC.
Fig. 3.15 Kinetics of priming to DNP after immunisation with DNP-CRBC.

Day 7(a) or 6(b) splenic PFC responses of groups of 5 irradiated (900r) recipients of $10^7$ pooled brachial and axillary lymph node cells and $10^8$ DNP-CRBCiv. Donors were immunised with $5 \times 10^7$ DNP-CRBCsc + $5 \times 10^8$ B.pertussis. IgM and IgG PFC responses ± 1 se are also given.

■·····, IgM PFC; ○——○, IgG PFC.
Days between priming and transfer

Graph a: Mean log$_{10}$ pFC± se vs Days between priming and transfer

Graph b: Mean log$_{10}$ pFC± se vs Days between priming and transfer

Legend:
- 1° IgM
- 1° IgG
Fig. 3.16. Kinetics of priming to DNP after immunisation with DNP-BGG

Day 6 splenic PFC response in groups of 5 irradiated (900r) recipients of $10^7$ pooled brachial and axillary lymph node cells and 50µg DNP-BGGiv. Donors were primed with 50µg DNP-BGG + $5 \times 10^8$ B.pertussis sc.

IgM and IgG responses ± 1 se are also given.

- - - - - , IgM PFC;   □□□□□□□□□ , IgG PFC.
Days between priming and transfer

mean log_{10} PFC±se

4 7 14 21

1^0 IgM

1^0 IgG
detected in one experiment at a time when there was a rise in another, and more work needs to be done to make the situation clearer.
DISCUSSION

1. Consideration of techniques

Adoptive transfer systems

In vivo transfer systems have proved a convenient and useful method of cell culturing. It is usually found that transferred lymphoid cells respond at least twice as well in X-irradiated recipients as after challenge in the original donor, and also that the response is often of lower affinity. This has been attributed to a release from normal regulation in the irradiated host rather than to stimulatory factors produced by it (Bell & Shand 1975). A linear relationship between the number of cells transferred and their antibody response in the recipient, has been described by several authors but only within a certain dose range (Celada 1967; Bosma et al 1968; Cunningham 1969a, b). Bosma et al (1968) showed this to be true for doses of SRBC-primed cells between $5 \times 10^6 - 1 \times 10^8$ and for unprimed cells between $10^7 - 10^8$. Doses of $10^7$ or $2 \times 10^7$ cells were chosen for the experiments in this work. The cell number transferred not only affects the level of the recipient response but also its character, though this is also influenced by the challenge antigen dose. Transfer of high cell numbers (more than $10^6$) or challenge with a low dose of antigen both bias the recipient response in favour of high affinity antibody, whilst low cell numbers and high antigen challenge induce a wide range of antibody affinities (Celada et al 1969; Macario et al 1973). The experiments in this chapter employed a high challenge dose of $10^8$ SRBC iv in an attempt to detect the complete range of clones represented. Since donor cells were pooled before transfer, where variability was seen within a recipient group, it probably reflected differences in lymphocyte homing to the
spleen perhaps due to damage of the cells. Less than 10% of transferred spleen cells actually colonise the spleen but it has been shown that these are the cells responsible for making antibody in the recipient (Playfair et al. 1965; Bosma et al. 1968; Askonas & Williamson 1972). Askonas and Roelants (1974) have recently shown that challenge with antigen-containing macrophages amplifies the response of transferred cells and they suggest that this may be due to increased efficiency in homing of T and B-cells to the same foci. Homing of lymph node cells may be less efficient than for spleen cells since Askonas and Williamson (1972) could not transfer the DNP clone, E9, with peripheral lymphocytes.

anti-IEF and PFC assays for SRBC responses

It has proved difficult to assess the sensitivity of the SRBC in agarose overlay for detection of electrofocused anti-SRBC antibodies despite the assertion of Phillips and Dresser (1973b) that it is an extremely sensitive assay. As well as sharp, clear bands there were always some extremely faint bands and often smears of lysis. The smears may represent small amounts of a wide range of antibodies but this is by no means certain. Different gels varied to some extent in sensitivity for faint bands, as seen when the same samples were run on different occasions. Conditions have been kept as near uniform as possible to minimise this, but it is necessary to be cautious of comparisons between samples run on different gels. Comparisons within a gel should be unaffected. One factor which profoundly reduced sensitivity was reduction in the time interval between SRBC overlay and addition of complement. A two-hour interval has been routinely used throughout these experiments since after this time sensitivity was good and diffusion not so great as to obscure the sharpness of the bands. Prominent bands can sometimes be traced between some, or all,
members of the recipient group implying that each recipient received memory cells of the same clone. Where unique bands were seen in individual members of a group, it implies that limiting numbers of some clones were transferred.

Both IEF and PFC assays are dependent on RBC lysis by guinea-pig complement and it would be expected that the results of the two assays would complement one another. A high IgG PFC response with a restricted number of high intensity bands would infer that relatively few, but well-expanded clones were involved in the response. On the other hand a high PFC response with a large number of low intensity bands (or smears) would indicate that a large number of poorly expanded clones were responding. In practice results have not been clear-cut and were often difficult to interpret. In some experiments the haemagglutination assay was also used to help distinguish between alternative explanations. The inconsistencies may have arisen from the use of different developing sera for each assay. The PFC developing serum was a rabbit anti-mouse IgG with specificity for all mouse IgG subclasses, but particularly for IgG1. This developing serum proved poor in the IEF assay so an anti-light chain serum has been used. It should thus detect all IgG classes efficiently.

Good correlations between the two assays were seen in some experiments, for example Figs. 3.1 and 3.2; Figs. 3.3 and 3.5a; Figs. 3.8a and 3.9a. However three major types of discrepancy between the two assays have been described in which cells primed three weeks before transfer gave:

a) equal PFC numbers but more intense spectra than more recently primed cells (Fig. 3.7a and Table 2)
b) equal PFC numbers but clonally restricted spectra compared
with more recently primed cells (Fig. 3.3 and Fig. 3.7b),
equal PFC numbers but less intense spectra than more recently
primed cells (Fig. 3.6 and Table 1; Fig. 3.10b and 3.11b;
Figs. 3.13a and b).

Askonas et al (1972) reported a situation in which PFC numbers
declined with time after adoptive transfer of cells despite continued
high antibody titres. They explain this by the residence of AFC
in organs other than the spleen and this explanation may also be
invoked for the first of the above situations. There is also the
possibility that day 21 primed cells produced more antibody per cell
than more recently primed cells. Neither of these explanations are
plausible since the HA titres (both total and 2-mercaptoethanol
resistant) were similar in recipients of day 7 and 21 primed cells.
There are also the possibilities that day 21 primed cells attained
an earlier peak response in recipients, that there was a IgG class
switch in the responding cell pool which went undetected for
technical reasons or that lower affinity antibodies were produced
by more recently primed cells. However since peak PFC responses
were usually on the same day for recipients of 7 and 21 day primed
cells (Fig. 3.1) and an anti-light chain developing serum should
have detected class switches, none of these explanations were
particularly satisfactory.

The observation that, despite high PFC levels on days 7, 10 and
14, clones became extremely restricted by days 10 and 14 was not
repeated and the possibility remains that it should be regarded
as a single untoward experiment. There was no increase in intensity
of restricted clones to indicate that a few clones became extremely
well-developed and could thus alone account for the PFC responses,
although it is possible that individual AFC produced less antibody.

The third category was a little more common than those discussed above, being seen in one experiment involving priming with SRBC and all CRBC experiments. These showed a high PFC response by cells from day 7, 14 and 21 primed mice despite less intense, but not clonally restricted IEF spectra from day 14 and 21 primed cells. Of the variables already discussed, reduced HA titres in the sera of recipients of day 14 or 21 primed cellssuggest that alterations in the kinetics of the adoptive response or in the output of antibody per cell could both account for this result. Very little is known about the factors affecting rate of antibody production at the level of the individual cell and it may well be that recently primed cells do make antibody at a faster rate. However the observation that day 7 anti-CREC-primed cells showed only a low PFC response but strong and diverse IEF spectra perhaps supports the concept of an earlier and transient peak 2° response for these cells. An alternative explanation for these observations is the existence of short-lived memory cells which are extremely efficient at antibody production (Strober 1976). However Strober has shown that these persist for several weeks after priming to DNP so they are unlikely to account for differences seen within the first 3 weeks of priming to RBC.

Bearing in mind reservations concerning the interpretation of these data, some useful information can be drawn from the results of experiments presented in this chapter.

2. Generation of memory to SRBC

IgG memory

Adoptive transfer of lymph node cells at intervals after sc priming with $5 \times 10^5$ or $5 \times 10^6$ SRBC, indicated that IgG memory to

83.
SRBC developed within 4-7 days of priming. The memory cell pool either reached a maximum size by the seventh day or enlarged for a further week, then maintained a constant size for the next few weeks. Other workers have studied the generation of memory to high and low doses of SRBC injected intraperitoneally or intravenously. It is difficult to correlate low \((5 \times 10^5 \text{ ip})\) and high \((5 \times 10^8 \text{ ip})\) doses for splenic priming with those for lymph node priming. An sc injection of \(5 \times 10^5\) SRBC probably reflects a 'low' dose since only a poor \(1^\circ\) antibody response is invoked, but a dose of \(5 \times 10^6\) induces a good \(1^\circ\) antibody response, particularly when incorporated with \(B. \text{pertussis}\). The adoptive \(2^\circ\) responses were similar whether priming was with \(5 \times 10^5\) or \(5 \times 10^6\) SRBC, or \(5 \times 10^7\) CRBC indicating that the priming antigen dose does not much affect memory generation to sc RBC. This is in accord with the results of Black & Inchley (1974) which showed that IgM and IgG B-cell memory developed in response to \(5 \times 10^5\) SRBC ip, but not with the results of others which only showed precursor memory after priming iv with high RBC doses (Cunningham & Sercarz 1971; Neiderhuber & Müller 1974). It would be useful to confirm that both T and B memory cells have been generated in the present experiments, perhaps by extending the hapten-carrier experiments or by adding purified T or B cells to the transferred cell suspensions.

There is some controversy over the later development of IgG memory to RBC antigen. The experiments in this chapter indicated that the capacity to give a secondary response reached peak levels between 1-2 weeks after priming and maintained those levels for the next few weeks. This observation is supported by the work of Black and Inchley (1974) who showed that IgG memory was fully developed within 7 days of priming and remained constant for the
next 11 weeks. However, Cunningham and Sercarz (1971) showed that precursor memory to cow RBC rises during the first nine days, remains constant for the next week then rises again over the next two months. They also showed that helper memory follows a similar pattern so this could not be regulating the response expressed in the present experiments. It is difficult to explain these contradictions, particularly since Cunningham and Sercarz (1971) also employed an adoptive transfer system to assay memory development, and used the same CBA strain employed by Black and Inchley (1974) and in the work reported here. It is also difficult to attribute differences to the use of different tissues since the results for lymph nodes support some observations for the spleen (Black & Inchley 1974) but not others (Cunningham & Sercarz 1971). Variations in RBC used are unlikely to have been important since in the present work the responses to SRBC and CRBC showed similar early kinetics. However the late CRBC response was not studied so it remains possible that different RBC persist for varying lengths of time and thereby affect the late memory response.

Clonal heterogeneity of the memory cell pool was assayed by IEF of antibodies produced in the adoptive secondary response. Memory was not always apparent in cells transferred four days after priming but, where seen, the clonal response was more restricted than at later times (Fig. 3.5a). This was not the case after priming with SRBC + B. pertussis, an adjuvant which probably affects the rate of cell division rather than recruitment (Chapter 5). It is possible that although memory cell recruitment was complete by day 4, memory clones were still expanding and that some were not sufficiently large to be detected by IEF after adoptive transfer. B. pertussis may increase the rate of this clonal expansion, thereby providing a
wide spectrum by day 4. The broad clonal spectra developed in cells transferred between 4-7 days after priming indicates that a wide range of clones had been recruited into the memory cell pool by that time. There was little change in the clonal spectra over the next few weeks indicating that there was no accumulation of memory clones due to continuing recruitment. It is likely that memory detected at 5-7 weeks after priming represents the longevity of individual memory cells recruited within the first week of priming and perhaps recycling in response to persistent antigen. This is in contrast to observations on the generation of memory to NIP (Kreth & Williamson 1973) during which new clones were recruited into the memory cell pool for 76 days after priming, although the pool had reached a maximum size within 30 days. Since the IEF spectra of adoptive 2° anti-SRBC antibody were heterogeneous, it is possible that clonal recruitment would not have been detected. If recruitment had continued for several weeks, then either the average number of B-memory cells per clone, or their capacity to divide to produce 2° APC after adoptive transfer, would have to fall to maintain 2° PFC at a constant level. There is no support for the former (Kreth & Williamson 1973) but there is some indication that recently primed memory cells divide more rapidly in vitro than do those primed for longer periods of time (North & Askonas 1976). It is also possible that with time after priming, low affinity memory clones become reduced in size due to competition for persistent antigen. Survival of high affinity clones would also imply that new clones must be of high affinity (Askonas et al 1976). The experiments here do not cover a sufficient time-scale to detect such differences.

IgM memory

A day 6 PFC assay was chosen for these experiments to show
peak memory IgG PFC at a time when $1^0$ IgG PFC were still low. However this did not prove a good day of assay for adoptive IgM responses since, although $1^0$ IgM PFC were at a peak, $2^0$ PFC were sometimes falling. A prompt response upon transfer is as much, or more, a feature of the $2^0$ response as are high levels of responsiveness. A day 4 or 5 assay would have been ideal for detection of IgM memory at a time when $1^0$ IgM responses were low.

There was an increase in the capacity to give an adoptive $2^0$ IgM response between days 2-4 (Fig. 3.3) after priming, and sometimes there was a further increase between days 4-7 (Fig. 3.4). This was probably a reflection of IgM memory generation but results must be viewed with some caution since the adoptive $2^0$ responses of some primed cells may be more transient than that of others (Fig. 3.1). Once the IgM memory cell pool reached a maximum size it either maintained that size for several weeks or fell to a lower level which was then maintained. There is no support for a reduction in IgM memory between days 9-16 after priming (Cunningham & Sercarz 1971; Black & Inchley 1974) or for a late rise in IgM memory (Cunningham & Sercarz 1971).

3. Generation of memory to CRBC

It was clear that memory generation to CRBC followed a similar pattern to that for SRBC, after priming with either CRBC or DNP $0.2^{CRBC}$. The capacity to give a $2^0$ IgG response upon adoptive transfer was well-developed 7 days after priming and remained high for at least 3 weeks. Broad clonal spectra were seen with cells primed 7 days before transfer and in some experiments the memory pool was well-developed within 4 days.

4. Generation of memory to DNP

Following priming with $5 \times 10^7$ DNP $0.2^{CRBC} + B. pertussis$, IgG
memory to DNP was first detected in cells transferred 21 days after priming. IgM memory rose from low levels at day 4 to a peak between days 7-14, then dropped slightly to a plateau. Since antigen-handling and T-cell priming were both similar for DNP$_{0.2}$CRBC and CRBC, as seen by comparing their ability to prime for a secondary response to CRBC, it would appear that the slower rate of priming to DNP is an intrinsic property of B-cell priming to the hapten itself. This theory is invalidated by the early generation of memory to DNP-BGG. IgG memory to DNP following priming with DNP-BGG, was high by day 7 and continued to increase during the next 2 weeks (Fig. 3.14c). The fall detected after day 7 in the second experiment (Fig. 3.16) was probably an artefact of the transfer system since no fall was seen after challenging donor mice in situ at 3 weeks after priming (Chapter 2). Mohr and Krawinkel (1976) have noted a decline in memory levels to BSA between 16 and 30 days after priming and adoptive transfer. One possible explanation for such an effect is the loss of short-lived memory cells but there is no good evidence for this. North and Askonas (1976) recently described an "intermediate" memory cell which could give an IgG response in vitro. These cells were found within 4-7 days of boosting, thus were derived from memory cells, and underwent division cycles in vitro before becoming AFC. Cells primed 4 days before culture gave a more rapid response than 7 day primed cells possibly because they did not need to undergo as many division cycles after challenge in vitro. They suggested that cells primed for longer periods of time may have to undergo even more divisions, thus $2^\circ$ IgG production by these cells could not be detected within the period of culture. It is possible that a day 6 assay in the experiments presented here, was too early to detect good anti-DNP memory to
DNP-BGG after transfer. A day 7 assay was used in the first experiment (Fig. 3.14).

Prior to performing these experiments, it was expected that memory generation to DNP would either be the same irrespective of the carrier molecule, or would occur earlier with an RBC than with a protein carrier. McMichael and Williamson (1974), using DNP-OA, have propagated an individual anti-DNP clone for several transfer generations and have shown that new memory cells are generated from pre-existing memory cells within 7 days of antigen boosting, though none could be detected after four days. This response within a single clone does not improve over the next 3 weeks. It is therefore likely that where a continued rise in memory generation normally occurs, it is due in large part to recruitment of new clones into the memory cell pool by pre-existing antigen (Kreth & Williamson 1973). It is thus not surprising that memory to DNP was detected 7 days after priming to DNP-BGG, although it would be interesting to extend the observations at later times to compare increases in PFC numbers with clonal spectra. The reasons for the unexpected late generation of memory to DNP after priming with DNP-CRBC remains obscure. Primed T-cells should be abundant and competition between the anti-CRBC and anti-DNP responses is unlikely. It is possible that memory B-cells were recruited into the primary response for the first 1-2 weeks after priming thereby depleting the anti-DNP memory cell pool but this is also unlikely since the 2° response to the carrier (CRBC) was unaffected. It would be interesting to extend these experiments beyond 3 weeks to see if the anti-DNP memory response attained a plateau, as for RBC, or continued to increase in levels.

In conclusion, in spite of the preliminary nature of some of
these experiments, it would seem that B-cell priming is independent of helper priming. Also, that it cannot be simply attributed to the soluble or particulate nature, but rather that it is an individual feature of specific particles or molecules.
CHAPTER 4

MEMORY IN A REGULATORY SYSTEM FOR THE ANTIBODY RESPONSE
INTRODUCTION

Immunological memory is characterised by the faster and generally larger response which follows challenge with the priming antigen. This is due, in part, to the presence of larger numbers of antigen-reactive cells, but also to intrinsic differences between virgin and memory cells. It is not yet known whether populations of primed regulatory cells also exist or whether primary and secondary responses are both under the control of primary regulatory cells. This question has been approached in the following study by attempting to prime the population of cells responsible for the phenomenon of immunological pre-emption.

Antigenic competition and pre-emption

The term "antigenic competition" refers to the reduced response to the second antigen when two antigens are injected sequentially, intraperitoneally or intravenously, within a few days of one another. Other forms of antigenic competition involving mutual competition of antigens injected together, or competition of the second antigen against the first, have been described (review: Liacopoulous & Ben-Efraim 1975). Studies have been extended to include competition between different draining lymph nodes (Fauci & Johnson 1971; Taussig 1971) and between spleen and lymph nodes (O'Toole & Davies 1971; Inchley et al 1975). The latter is more commonly referred to as immunological pre-emption, and was first described as suppression of the PFC response to subcutaneous injection of SRBC caused by intraperitoneal injections of HRBC or SRBC four days previously (O'Toole & Davies 1971). The experiments have now been extended to include several other antigen pairs (Black 1973). Black (1973) has shown pre-emption of an SRBC
response by rat RBC and chicken RBC but not by allogeneic mouse RBC, T2 phage or SIII polysaccharide. However it is unlikely that pre-emption only exists between RBC antigens since lymph node competition has been demonstrated between the haptens TNF and Ars (arsanilic acid) (Fauci & Johnson 1971), and between Fc and F (ab)2 antibody fragments (Taussig 1971). Splenic competition has been shown between many protein and RBC pairs (Liacopoulous & Ben-Efrain 1975), for example ferritin and BGG in mice (Adler 1964), KLH and BSA in rabbits (Cremer 1963), rat RBC and goose RBC in mice (Eidinger et al. 1968), hemacyanin and rat or goose RBC (Eidinger et al. 1968). It has been suggested that the degree of pre-emption may be related to the immunogenicity of the first antigen (Black 1973); a reasonable explanation for lack of pre-emption by allogeneic mouse RBC in mice since they elicit only poor responses.

Antigenic competition and pre-emption may both be a reflection of inhibition of cell recruitment, or proliferation, in response to the second antigen, by a regulatory stage in response to the first (Pritchard-Briscoe et al. 1977). It has been suggested that in pre-emption the regulatory stage of an immune response may be isolated in space from the other events and therefore more easily analysed. Despite this, there is no obvious explanation for pre-emption. At least four current theories exist and one, or all, of them may be involved. The last three would also adequately explain most forms of antigenic competition.

1) Cells are trapped in the spleen by an efferent pathway block set up in response to the intraperitoneal injection. This would make them unavailable for a response in the lymph nodes at the time of challenge.
A substance, made in the spleen in response to the first antigen, prevents lymphocytes from entering into the spleen and nodes for response to the second antigen.

There is competition between T-cell helper factors, at the level of the macrophage (Taussig & Lachman 1972; Feldmann & Schrader 1974).

A substance, made in the spleen in response to the first antigen, "switches off" all lymphocytes so that specific cells are unable to respond to subcutaneous challenge (Redelman et al 1976; Pritchard-Briscoe et al 1977).

The first explanation is perhaps the least likely. Although cells of all specificities are trapped in lymphoid organs after antigen localisation (Zatz & Lance 1971), the work of Sprent has shown that only specifically responding cells are completely removed from the circulating pool by antigen in the spleen (Sprent et al 1971; Sprent 1973). This explanation could thus only apply to pre-emption caused by two sequential injections of the same antigen. Sprent's findings also raise an objection to the second theory, since recovery of HRBC reactive cells in the thoracic duct lymph of SRBC immunised mice implies that near-normal circulation of lymphocytes was taking place through the lymph nodes. On the other hand, Black (1973, 1975) has shown that a reduction of lymphocyte entry into peripheral lymph nodes can follow intraperitoneal immunisation with SRBC, and it remains possible that their circulatory behaviour is modified. One way in which this may be accomplished is by binding of a substance such as phospholipase A, which is released by activated macrophages, and which reduces lymphocyte passage through the lymph nodes (Freitas & de Sousa 1976a and b).
However, binding of phospholipase A could not be an explanation for competition since treated lymphocytes tend to accumulate in the spleen.

The most persuasive explanations for antigenic competition involve direct T-lymphocyte activity, either by the production of saturating levels of IgT (Taussig & Lachman 1972; Feldmann & Schrader 1974), or by the synthesis of regulatory factors by suppressor cells (Redelman et al 1976). Either of these could account for pre-emption provided that the factors involved were produced in sufficient amounts to give an adequate concentration in the lymph nodes. However it is difficult to interpret the observation that recirculating but not sessile memory cells are susceptible to pre-emption (Inchley et al 1975) in terms of these theories, unless the latter cell types are resistant to pre-emption by virtue of their position in the nodes, or their relationships with other cells.

If pre-emption results from the activity of a regulatory factor, as has been proposed for antigenic competition (Thomas et al 1975; Redelman et al 1976; Pritchard-Briscoe et al 1977), then it can be attributed to the activity of suppressor T-cells (Gershon & Kondo 1971). However it is difficult at present to correlate the information on possible T-cell effects in pre-emption and competition with current knowledge on suppressor T-cells. Adult thymectomy reduces antigenic competition (Liacopoulous & Ben-Efraim 1975) and reduces suppressor cell activity (Basten et al 1975), as does splenectomy (Wu & Lance 1974; Gershon et al 1974).

These observations implicate the importance of the short-lived T-cell in both competition and suppression, but adult thymectomy has
no effect on pre-emption (Black 1973). Similarly, suppressor cells are activated by the T-independent antigen SIII (Baker 1975) but immunisation with SIII failed to produce pre-emption.

Murine suppressors are characterised by their surface Ly antigens (Herzenberg et al 1976) and their irradiation sensitivity (Chan & Henry 1976). Apart from cells stimulated non-specifically by mitogens such as CON A (Redelian et al 1976), two categories of suppressor cell have been described; those with specificity of action and those whose activity affects a broad spectrum of responses. Only the latter is likely to function in antigenic competition or pre-emption.

The observation that secondary responses compete better than primary provides reason for speculation on the existence of a primed suppressor T-cell population (Eidinger et al 1968; Moller & Sjoberg 1970). Very few studies have involved deliberate priming of suppressor T-cells although some utilise 2-3 injections to raise a good population of suppressors (Sjoberg & Britton 1972; Tada et al 1975) and others include antigen in culture to activate suppressors in primed cell populations (Thomas et al 1975). However Benacerraf et al (1975) could not demonstrate memory in a population of suppressors raised to GAT (L-glutamic acid-L-alanine-L-tyrosine).

Aim of study

The following study has involved an attempt to further characterise the cells which are responsible for immunological pre-emption by priming them and by comparing the kinetics of priming with those for helper T-cells. The results are discussed with reference to pre-emption being caused by helper T-cells (Feldmann & Schrader 1974; Taussig & Lackman 1972) and/or suppressor T-cells (Gershon & Kondo 1971; Redelman et al 1976).
MATERIALS AND METHODS

1. Animals, antigens and PFC assay were as described in Chapter 3.

2. Measurement of DNA synthesis by incorporation of \(^{125}\text{I}-\text{iodo-2-deoxyuridine (}^{125}\text{I-UdR)}\)

\(^{125}\text{I-UdR (Radiochemical Centre, Amersham)}\) was adjusted immediately before use to a specific activity of 5\(\mu\)Ci and a concentration of 1.9\(\mu\)g, by addition of cold IUdR. Mice were injected ip with 0.2 ml saline containing \(5 \times 10^{-8}\) moles 5-fluorodeoxyuridine (FUdR - Roche Productions Ltd.) followed after 1 hour by 1\(\mu\)Ci \(^{125}\text{I-UdR by the same route, and were killed 2 hours later (Pritchard & Micklem 1972).}

Lymphoid tissues to be assayed were excised and placed in 70% ethanol. They were washed in several changes of ethanol until the wash was free of radioactivity. Tissues were then counted for 2 minutes in a \(\gamma\)-counter (Nuclear Enterprises). The activity of \(^{125}\text{I-UdR injected into each animal was determined and the organ counts per minute (cpm) were corrected against an arbitrary standard of 10}^6\) as follows

\[
\text{corrected cpm} = \frac{\text{actual cpm} \times 10^6}{\text{dose cpm}}
\]

The activity in each organ is expressed as \(\log_{10}\) corrected cpm.
**RESULTS**

The pre-emptive effect of a primary response

Pre-emption of a primary lymph node response is best achieved by ip injection of high doses of antigen 2-4 days before sc test immunisation (O'Toole & Davies 1971). This was confirmed for CRBC pre-emption of the SRBC response (Fig. 4.1) and a 2 day interval was chosen for subsequent experiments. In priming experiments, injections of the suppressing antigen were given at intervals up to 3 weeks before ip challenge (= 23 days before sc immunisation). It was therefore important to establish that no residual suppressor effect was evident 23 days after an ip injection of the suppressive antigen (Table 1). It can be seen that there was no pre-emption of the response to sc SRBC or DRBC as a result of either high (5 x 10^8) or low (10^6) doses of CRBC given 23 days beforehand. These results may be compared with the inhibition of the response which followed a single pre-empting injection of CRBC 2 days before test immunisation with SRBC or DRBC, when IgM responses were reduced at least 100 fold and IgG often as much as 1000 fold (Fig. 4.2).

DRBC were equally as effective as CRBC in pre-empting the response to SRBC but pre-emption of a response to CRBC was less pronounced although still significant (Fig. 4.3). Background responses to CRBC were not included and may have been high. Again there was no difference in the anti-SRBC response given by normal mice and those injected with 10^6 or 5 x 10^8 DRBC ip 23 days previously. The latter (receiving 5 x 10^8 DRBC ip) are shown as controls in Fig. 4.3.

The pre-emptive effect of a secondary response

Priming with 10^6 or 5 x 10^8 RBC ip 3 weeks prior to pre-emption with the same antigen failed to increase the level of suppression
4.1 Pre-emption of the lymph node response to SRBC by a high dose of CRBC.

Groups of 5 mice were injected with $5 \times 10^8$ CRBCip2 or 4 days prior to immunisation with $5 \times 10^7$ SRBCsc. Brachial lymph nodes were assayed vs SRBC on day 5. Results are expressed as mean log$_{10}$ PFC per brachial lymph node pair.
TABLE 1

Failure of CRBC to pre-empt a response to RBC injected sc 23 days later

<table>
<thead>
<tr>
<th>CRBC</th>
<th>Test antigen</th>
<th>IgM PFC mean log_{10} ± se</th>
<th>IgG PFC mean log_{10} ± se</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>DRBC</td>
<td>2.93 ± 0.10</td>
<td>3.77 ± 0.16</td>
</tr>
<tr>
<td>10^6</td>
<td>DRBC</td>
<td>3.12 ± 0.10</td>
<td>4.03 ± 0.17</td>
</tr>
<tr>
<td>5 x 10^8</td>
<td>DRBC</td>
<td>2.83 ± 0.15</td>
<td>3.81 ± 0.13</td>
</tr>
<tr>
<td>-</td>
<td>SRBC</td>
<td>4.19 ± 0.08</td>
<td>2.62 ± 0.88</td>
</tr>
<tr>
<td>10^6</td>
<td>SRBC</td>
<td>4.50 ± 0.08</td>
<td>1.00 ± 1.00</td>
</tr>
<tr>
<td>5 x 10^8</td>
<td>SRBC</td>
<td>4.28 ± 0.08</td>
<td>0.92 ± 0.92</td>
</tr>
</tbody>
</table>
Fig. 4.2 The effect of priming on the degree of pre-emption induced by CRBCip.

A, day 5 10^7 PFC response to 5x10^7 SRBCsc(a and b) and DRBCsc(c).
B, pre-empted with 5x10^8 CRBCip 2 days prior to test SRBC or DRBC injection.
C, primed with 10^6 CRBCip 21 days before pre-emption.
D, primed with 5x10^8 CRBCip 21 days before pre-emption.

Results are expressed per brachial lymph node pair.

|   | IgM PFC;   | IgG PFC. |
a. 

b. 

c.
Fig. 4.3 Pre-emption of the lymph node response to SRBC and CRBC by $1^0$ and $2^0$ responses to DRBC.

A, day 5 $1^0$ PFC response to $5 \times 10^7$ SRBC(a) or CRBC(b).

B, pre-empted with $5 \times 10^8$ DRBCip 2 days before test SRBC or CRBC injection.

C, primed with $10^6$ DRBCip 21 days before pre-emption.

D, primed with $5 \times 10^8$ DRBCip 21 days before pre-emption.

E, background response to SRBCsc.

Results are expressed per brachial lymph node pair.

\[ \text{IgM PFC; } \text{IgG PFC.} \]
(Fig. 4.2 and 4.3) and even reduced it in one experiment (Fig. 4.2a), a result which is difficult to explain since it was not consistently observed. High dose \((5 \times 10^8)\) pre-emption was so pronounced that it would be difficult to observe any great improvement resulting from priming. Accordingly, a lower antigen dose \((10^6 \text{ RBC})\) was chosen which usually slightly depressed the response to the test antigen but not to a significant degree (Fig. 4.4 a, b, c). It was hoped that, by choosing a dose which was borderline in ability to pre-empt on its own, it might be possible to detect suppressor cell priming. The effect of a priming dose of \(10^6\) DRBC before low dose pre-emption was also studied in these experiments, and was found to give a significant degree of pre-emption of the IgM response after a priming interval of 2 days \((P < 0.05)\), 7 and 21 days \((p < 0.01)\).

Onset of \(1^\circ\) IgG response was variable, giving large standard errors and making it difficult to study pre-emption of IgG production despite evident differences in mean PFC values. Since the effect of pre-emption on the IgM response is masked by declining PFC levels at later times, it was decided to continue with the day 5 assay, but confine the study to IgM only.

These results were confirmed by three more experiments in which a low dose of ip DRBC two days prior to SRBC so failed to significantly reduce the anti-SRBC PFC response unless preceded by a priming injection 7 or 14 days beforehand (Fig. 4.5). Priming 2 days prior to low dose pre-emption had variable effects - in two experiments it failed to produce suppression but in the third (Fig. 4.5b) pre-emption was equally as good as that obtained with a 7 day interval.

Low dose pre-emption by CRBC was not improved by prior priming with \(10^6\) CRBC ip (Fig. 4.6) but it is possible that \(10^6\) CRBC is not
Fig. 4.4 Pre-emption of the 1st response to SRBC by a low dose of DRBC and the effect of priming on the degree of pre-emption

Groups of 5 mice were injected with $5 \times 10^8$ (B) or $10^6$ (C) DRBCip 2 days prior to immunisation with $5 \times 10^7$ SRBCsc. Group (A) received $5 \times 10^7$ SRBCsc only. Group (D) were primed with $10^6$ DRBCip 2(a), 7(b) or 21(c) days prior to the pre-emptive injection of DRBCip.

Day 5 PFC responses vs SRBC are expressed as mean log$_{10}$ PFC per brachial lymph node pair.
Fig. 4.5  The effect of priming on the degree of pre-emption  
effected by a low dose of DRBCip  

Groups of 5 mice were injected with $5 \times 10^8$ (B) or $10^6$ (C) DRBCip 2  
days prior to immunisation with $5 \times 10^7$ SRBCsc. A, received SRBC only.  
D, E and F were primed with $10^6$ DRBCip 2(D), 7(E) or 14(F) days  
prior to low dose pre-emption. Day 5 PFC responses vs SRBC are  
expressed as $\log_{10}$ mean PFC per brachial lymph node pair.
Fig. 4.6 The effect of priming on the degree of pre-emption
affected by a low dose of CRBC.
Groups of 5 mice were injected with $5 \times 10^8$ (B) or $10^6$ (C) CRBCip 2
days prior to immunisation with $5 \times 10^7$ SRBCsc. A, injected with SRBCsc
only; D, primed with $10^6$ CRBC ip 2 days prior to pre-emption; E,
primed with $10^6$ CRBCip 7 days prior to pre-emption. DAY 5 PFC
responses vs SRBC are expressed per brachial lymph node pair.
mean $\log_{10} \text{IgM PFC} \pm \text{se}$

A
B
C
D
E
sufficiently high to represent a borderline pre-empting dose for this antigen.

Assay of dividing cells by uptake of $^{125}$IudR has proved useful for demonstrating pre-emption (Black 1973), proliferation to the test antigen being reduced at both day 3 and 5 of assay. These results were confirmed (Fig. 4.7a and b) and in addition it was shown that low dose pre-emption had no significant effect on $^{125}$IudR incorporation on either day. However a significant degree of pre-emption ($p < 0.01$) was induced by a low dose of DRBC following priming 7 days previously with $10^6$ DRBC ip. Priming 14 days previously did not increase this effect and day 2 priming was borderline, significantly lower than the low dose pre-emption control on day 3 in one experiment (4.7a) and day 5 in the other (4.7b).

Using the same scheme for suppression, an experiment was done to see if these priming effects could be observed in antigenic competition (Table 2). $5 \times 10^8$ DRBC or $10^6$ DRBC were injected ip 2 days prior to $5 \times 10^8$ SRBC ip. The degree of competition seen even after the high dose was poor, possibly because the intraperitoneal rather than intravenous route was used and a 2 day rather than 4 day interval chosen (Radovich & Talmage 1967). No competition was seen after a single dose of $10^6$ DRBC ip or after two injections 2 days apart, but both 7 and 21 day priming induced significant competition ($p < 0.01$), in this case as good as high dose competition.

It was decided to try and improve the degree of pre-emption obtained with $10^6$ DRBC ip, firstly by giving multiple priming injections of $10^6$ DRBC ip. Two or three priming injections at weekly intervals failed to improve the degree of pre-emption beyond that obtained after one priming injection only given 1-3 weeks prior
Fig. 4.7 Measurement of pre-emption by incorporation of $^{125}$IUDR

Groups of 5 mice were injected with $5 \times 10^8$ (B) or $10^6$ (C) DRBCip 2 days before immunization with $5 \times 10^7$ SRBCsc. A, received SRBCsc alone; D, E and F were primed with $10^6$ DRBCip 2(D), 7(E) and 14(F) days before pre-emption. Day 3 and 5 results of an $^{125}$IUDR assay are expressed as mean log$_{10}$ cpm incorporated per brachial lymph node pair.
<table>
<thead>
<tr>
<th>Dose</th>
<th>Day</th>
<th>DRBC</th>
<th>Mean log$_{10}$ PFC$^{\pm}$se</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>$5.33 \pm 0.11$</td>
</tr>
<tr>
<td>$5 \times 10^8$</td>
<td>0</td>
<td>-</td>
<td>$4.76 \pm 0.11$</td>
</tr>
<tr>
<td>$10^6$</td>
<td>0</td>
<td>-</td>
<td>$5.25 \pm 0.07$</td>
</tr>
<tr>
<td>$10^6$</td>
<td>2 &amp; 0</td>
<td>-</td>
<td>$5.12 \pm 0.08$</td>
</tr>
<tr>
<td>$10^6$</td>
<td>-7 &amp; 0</td>
<td>-</td>
<td>$4.61 \pm 0.05$</td>
</tr>
<tr>
<td>$10^6$</td>
<td>-21 &amp; 0</td>
<td>-</td>
<td>$4.70 \pm 0.08$</td>
</tr>
</tbody>
</table>

Day 7 Splenic PFC response to $5 \times 10^8$ SRBC injected ip on day 2. DRBC were injected ip at various intervals before SRBC.
Fig. 4.8 The effect of multiple priming injections of DRBC on the degree of low dose pre-emption

Groups of 5 mice were injected with $5 \times 10^8$(B) or $10^6$(C) DRBCip 2 days before immunisation with $5 \times 10^7$ SRBCsc. A received SRBCsc only; D, E, F and G were primed with $10^6$ DRBCip on day 7(D), 7 and 14(E), 7, 14 and 21(F), or day 21(G) before pre-emption. Day 5 PFC responses vs SRBC are expressed as mean $\log_{10}$ IgG PFC per brachial lymph node pair.
mean $\log_{10} \text{IgM PFC} \pm \text{se}$
Fig. 4.9 The effect of priming with a high dose of DRBCip on the degree of pre-emption effected by a low dose.

Groups of 5 mice were injected with $5 \times 10^8$ (B) or $10^6$ (C) DRBCip 2 days before immunisation with $5 \times 10^7$ SRBCsc. A received SRBCsc only; D were primed with $10^6$ and C with $5 \times 10^8$ DRBCip 21 days prior to low (C) or pre-emption. Day 5 IgM PFC responses are expressed per brachial lymph node pair.
to low dose pre-emption (Fig. 4.8). The second approach was to prime mice ip with $5 \times 10^8$ DRBC three weeks prior to low dose pre-emption (Fig. 4.9). Again there was virtually no improvement in the degree of pre-emption - it closely resembled the reduction seen previously after low dose priming. These experiments indicate that the challenge dose may be insufficient to activate primed cells.

**Kinetics of priming with low antigen doses**

The kinetics of priming for a splenic PFC response to challenge with $5 \times 10^8$ DHBO were studied and compared with those for priming for low dose pre-emption. Two experiments are shown in Fig. 4.10, and in both it can be seen that priming with $10^6$ DRBC ip 2 days prior to challenge with $5 \times 10^8$ DRBC ip raised the splenic IgM response to DRBC above primary levels. Day 7 priming increased it further and also significantly raised the IgG response (Fig. 4.10b). One injection 3 weeks previously had no effect and neither did a series of 2-3 priming injections of $10^6$ DRBC ip. These kinetics closely follow those seen for low dose pre-emption and may be a reflection of T-cell helper priming only, since it has been reported that iv injections of low doses of RBC ($< 2 \times 10^7$) have a minimal priming effect on B-cells (Cunningham & Sercarz 1971; Neidehüber & Möller 1974). However others have shown good B-cell priming in response to $5 \times 10^5$ SRBC ip (Black & Inchley 1974).

"Helper" priming was not obvious after a low DRBC challenge probably due to the inability of this dose to stimulate B-cells to more than a low response, barely above background (Table 3).
Fig. 4.10 The effect of low dose priming on the splenic PFC response to DRBC.

Groups of 5 mice were immunised with $5 \times 10^8$ (A) DRBC ip and splenic PFC responses assayed 5 days later. Groups B, C, D and E were primed with $10^6$ DRBC ip at intervals before challenge with $5 \times 10^8$ DRBC ip:

Expt. (a)
B, 2 days; C, 7 days; D, 21 days; E, 7, 14 and 21 days.

Expt. (b)
B, 2 days; C, 7 days; D, 2 and 7 days.
<table>
<thead>
<tr>
<th>Primary and secondary PFC responses to $10^6$ DRBC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>day -7</strong></td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>-</td>
</tr>
<tr>
<td>-</td>
</tr>
<tr>
<td>$10^6$</td>
</tr>
<tr>
<td>-</td>
</tr>
<tr>
<td>$10^6$</td>
</tr>
<tr>
<td>-</td>
</tr>
</tbody>
</table>

**TABLE 3**
DISCUSSION

These experiments confirm the observations of others (O'Toole & Davies 1971; Black 1973; Inchley et al. 1975) that an intraperitoneal injection of foreign RBC pre-empts the response to a subsequent subcutaneous RBC injection. This was demonstrated both by reduced numbers of PFC and reduced proliferation (as measured by incorporation of $^{125}$IUDR) in response to the test antigen. The theories for pre-emption considered in the introduction propose that it is effected by one of the following:

1) production of an anti-mitotic agent (either by T-cells or T-cell activated macrophages) in a regulatory stage of the response to the first antigen;

2) production of a substance which prevents lymphocyte entry into the lymph nodes either by way of an afferent pathway block or by affecting migration of recirculating cells;

3) competition for macrophage sites between IgT produced in response to the first and second antigens.

Of these three the latter invokes helper rather than suppressor cell activity.

Observations on the resistance of sessile but not recirculatory memory cells to pre-emption (Inchley et al. 1975) allow some discrimination between these theories. This is best explained by prevention of lymphocytes from entering the lymph node, but other observations on the responses of pre-empted lymph node cells in adoptive recipients have indicated that they contain a normal complement of cells (Traill 1973). Release from suppression by adoptive transfer could be adequately explained by both competition...
between helper factors and by production of suppressor factors. Competition between IgTs should equally affect primary and secondary responses since both are T-cell and macrophage dependent (Miller & Sprent 1971; Oppenheim & Rosenstreich 1976), thus it is unclear why sessile memory cells escape pre-emption. Likewise, although memory cells may have a higher threshold for suppressor factors than virgin cells (Gershon 1975) and thus might be less affected by pre-emption, it is not easy to conceive of a differential between sessile and recirculating cells. In both situations it may be possible for sessile memory cells to be protected to some extent from circulating factors due to their position within the lymph nodes.

It is obviously difficult to explain all information on pre-emption by any one of the above theories, thus the results of the experiments presented in this Chapter will be discussed with respect to both helper and suppressor cells.

**High dose pre-emption**

The degree of pre-emption produced by a dose of $5 \times 10^8$ DRBC ip was so great as to reduce responses almost to background levels and make it impossible to detect further decrease due to priming. Since a single injection of DRBC ip 23 days before so SRBC failed to pre-empt it must be concluded either that the cells responsible quickly disappear and a new population is generated by each antigen injection, or that they are represented by a memory population which requires restimulation for expression. Suppressor cells may be expected to be still active at this time (23 days) since the RBC response is still continuing at low levels (see Chapter 5) and others have demonstrated suppressors active 28 days after antigen injection (Benacerraf et al 1975). However if pre-emption is due to
IgT competition then the first possibility is ruled out, since the presence of primed helper cells is well established.

**Low dose pre-emption**

A low dose of $10^6$ DRBC ip slightly suppressed responses to a subsequent challenge with SRBC but never to a significant degree. Only a poor immune response developed to such a low antigen dose so its inability to pre-empt could be explained by insufficient stimulation of help, resulting in non-competing levels of IgT. Although it is known that low doses of antigen prime T-cells well (Cunningham & Sercarz 1971; Black & Inchley 1974), it is uncertain whether they also provide a good stimulus for 'help'. If they do, the IgT production would be the same for high or low antigen doses, and thus pre-emption should also be the same after each. On the other hand, if suppressor cells are generated by the pre-empting injection, then their activity is probably related to the level of proliferation which results from ip immunisation (Pritchard-Briscoe et al 1977). Anti-proliferative factors have been isolated from both Con A stimulated (Redelman et al 1976) and OVA stimulated (Thomas et al 1975) spleen cells. It follows that in a weak proliferative response, such as that induced by $10^6$ DRBC, only low levels of suppressor factors would be required thus it is possible that the poor suppression of a test response to SRBC after low dose pre-emption may result from a minimal stimulation of suppressor cells. A high dose of antigen would induce many suppressors to control the high proliferative response and thus cause excellent pre-emption.

**The effect of priming on low dose pre-emption**

A priming regime shown to be effective for helper T-cell priming (Cunningham & Sercarz 1971; Black & Inchley 1974) but
possibly not for B-cell priming (Cunningham & Sercarz 1971) was chosen for these experiments. Confirmation of low dose priming was obtained by studying the secondary response after challenge with $5 \times 10^8$ DREC ip. Priming was slight by day 2, increased by day 7 but was not improved by longer intervals of time or by multiple injections. The same conditions of priming were utilised for the induction of both pre-emption and competition and were found to show very similar kinetics to those seen for the antibody response. This observation does not necessarily implicate the helper T-cell as being responsible for pre-emption and competition although it is compatible with the IgT theory. It is likely that T-suppressor activity is closely affected by helper activity since they act together to control antibody production and it would not be surprising if all T-cells were to follow similar priming kinetics.

In conclusion it does seem that both helper T-cells and those causing pre-emption are primed within 2-7 days of a low dose of antigen. The establishment of a closer relationship between the two cell types was not possible because of the limitations of the systems used, namely because challenge régimes optimum for the detection of T and B cell memory are not appropriate for pre-emption.

It remains possible that the two cells are one and the same, and that pre-emption (and competition) are mediated by excess of helper factor according to the scheme of Feldmann & Schrader (1974). The experiments reported here do not allow a conclusion to be drawn as to whether suppressor cells are involved. Should this prove to be the case, then it is of interest that they can be primed and that priming follows the same kinetics as that for help. The key questions in clarifying the activity of the primed pre-emption-
causing cells hinge on the degree to which low antigen doses
i) stimulate IgT production or ii) recruit suppressors from the
available pool. If two cell types are shown to be involved then a
comparative study of their activation as well as their kinetics of
priming would be of interest.
CHAPTER 5

The adjuvant effects of Corynebacterium parvum and
Bordetella pertussis on the response to SRBC
INTRODUCTION

An adjuvant can be defined as any agent that acts non-specifically to increase an immune response to a specific antigen (Allison 1973). This effect is often measured in terms of raised levels of antibody production though some adjuvants can enhance the cell-mediated immune (CMI) response. In this section only the effects of adjuvants upon the cells of the immune system will be considered since physical "depot" effects of adjuvants such as alum and Freund's complete adjuvant (FCA) are not relevant to the action of bacterial adjuvants used in this study. Dresser (1973) summarised the possible effects of an adjuvant on lymphoid cells as follows:

1) It would increase the probability of close contact between the cell types involved in the immune response.
2) It might improve the presentation of antigen to antigen-sensitive cells.
3) It might supply a "second signal" to B-cells for antibody production.
4) It might increase the pool of antigen-sensitive cells.
5) It could increase the rate of proliferation of antigen-induced cells and consequently also of entry of cells into the pool of antibody producers.
6) It could stimulate the production of positive feedback factors.
7) It could increase the rate of production of antibody or the productive life of antibody producing cells (AFC).

It has become apparent that different adjuvants act in different ways and that many have more than one effect on lymphoid cells. Most adjuvants are effective in increasing the localisation of recirculating lymphocytes (trapping) and thus in increasing the
probability of interactions between antigen-sensitive cells. This is particularly true of the 'strong' adjuvants such as complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), Corynebacterium parvum, Bordetella pertussis and Vitamin A but also of carbon and silica which are weak adjuvants (review Frost & Lance 1973). Increased trapping may be the result of a potent stimulation of the reticuloendothelial system (RES) by adjuvants (Halpern 1974) since the mechanism is believed to be macrophage dependent (Frost & Lance 1974). Another effect of RES stimulation is the altered handling and release of antigen by macrophages. Uptake of radiolabelled KLH (125I-KLH) was slower by C. parvum stimulated macrophages than by normal macrophages and more was retained on the membrane of C. parvum macrophages (Wiener & Bandieri 1975). This may explain the ability of adjuvants to enhance the response to subimmunogenic doses of antigen.

Some adjuvants have been shown to be mitogenic for lymphocytes. For example lipopolysaccharide (LPS) is mitogenic for murine B-cells (Schmidtke & Dixon 1972). They are believed to bypass the T-cell by providing the necessary second signal for antibody production (Bullock & Anderson 1973). Others, such as poly(Apolar)U and lentinan, are mitogenic for T-cells and may amplify the helper T-cell compartment (Allison 1973). In the absence of antigen it is possible that both T and B-cell mitogens could increase the pool of antigen-sensitive cells but, coupled with antigen stimulation, the effect may be more pronounced on antigen-specific cells (Dresser & Phillips 1973).

The influence of adjuvants on the antibody response can be seen at the cellular level. Biozzi et al (1966) demonstrated an increase
in antibody output per rosette forming cell after C. parvum treatment. One group (Tanaka et al 1975) measured the increase in plaque forming cell (PFC) diameter to assess the adjuvant effect of wax D (from Mycobacterium tuberculosis) on the response to SRBC. They found this more striking than the increase in PFC numbers, and have shown that the adjuvant is selecting a population of precursor cells capable of producing large PFC (Ishibashi et al 1975; Kohashi et al 1975).

There is little doubt that in some way feedback control of antibody responses is abrogated after adjuvant treatment, since antibody production persists at higher levels and for longer periods of time. However it remains pure speculation as to whether this abrogation results from increased helper signals, reduced feedback and suppressor signals or reduced susceptibility to the latter. Increase in the longevity of individual cells may add to this effect but cannot completely explain it.

The Adjuvant Effects of C. parvum

Corynebacteria can depress certain T-cell mediated immunological reactions such as the Graft versus Host (GvH), mixed lymphocyte culture (MLC) and the phytohaemagglutinin (PHA) responsiveness of spleen cells (Howard et al 1973; Toujas et al 1973). Graft rejection in vivo can also be delayed (Woodruff & Dunbar 1973; Ruitenberg & Steerenberg 1973). This contrasts with the potent adjuvant effect of these organisms on many antibody responses (Howard et al 1973b; Wiener 1975; Warr & James 1975; Watson & Sljivic 1976).

Most adjuvants are only effective in potentiating the antibody response to T-dependent antigens and are thought to act at the level of T-B cooperation, probably by expanding T-cell help. C. parvum
is, however, effective in potentiating the antibody response to the T-independent antigens SIII (Howard et al. 1973a), DNP-levan and DNP<sub>50</sub>EGG (which has many characteristics of a T-independent antigen) (del Guercio & Leuchars 1972) although not to the T-independent antigen DNP-PCL (Sijivic & Watson 1977). The latter discrepancy in results is not clearly understood although it is possible that T-independent antigens differ with respect to macrophage handling (Oppenheim & Rosenstreich 1976; Lee et al. 1976) and T-cell regulation of responses (Baker 1975).

The effect of C. parvum on T-independent antigens is not likely to result simply from its mitogenic action on B-cells since Corynebacteria lacking adjuvanticity were shown to possess mitogenic activity for B-cells (Zola 1975). In addition, Howard et al. (1973b) observed that although C. parvum treatment could enhance the anti-SRBC response of adult thymectomised, irradiated and bone marrow reconstituted (ATXI) mice to levels comparable to those of normal mice treated with C. parvum. Dresser and Phillips (1973) derived a formula to determine whether adjuvants are predominantly orientated towards T or B-cells. This involves a comparison of the ratio of IgM to IgG1 at different antigen doses and was applied by Warr and James (1975) to the adjuvant effect of C. parvum on the response to SRBC. They concluded that C. parvum activity was directed towards both T and B cells. Despite this the IgM, IgG2a and IgG2b antibody classes were more enhanced than the IgG1 class which is the most T-dependent.

Both the enhancing and suppressive effects of C. parvum have been attributed to the action of activated macrophages (Howard et al. 1973a and b; Watson & Sijivic 1976). It is a remarkably potent
stimulant of the RES, causing marked macrophage proliferation (Warr & Sljivic 1974c) and increased rates of carbon clearance (Halpern et al. 1964; Adlam & Scott 1973; O'Neill et al. 1973). Maximum adjuvant effects were found 4 - 7 days after an intravenous injection of \textit{C. parvum} (Sljivic & Watson 1977). It is at this time that delayed hypersensitivity (DH) to a subcutaneous injection of \textit{C. parvum} is at its peak (Tuttle & North 1975). Macrophage activation by \textit{C. parvum} requires interaction with T-cells (Sljivic & Watson 1977).

The activation of peritoneal exudate (PE) macrophages by an intravenous injection may be explained by the circulation of sensitised T-cells since very little \textit{C. parvum} reaches the peritoneal cavity by this route (Sadler et al. 1977). Assuming the importance of macrophage activation, it is difficult to explain the action of \textit{C. parvum} in ATxBM mice unless either residual T-cells are activated or there is an alternative mechanism for direct macrophage activation. \textit{C. parvum} binds to the plasma membrane of macrophages and may directly activate them in this way (Ogmundsdottir & Weir 1976).

Altered antigen handling by \textit{C. parvum} activated macrophages may result in more efficient antigen presentation to the lymphocytes. There is some evidence for this from the observation that \textit{C. parvum} macrophages took up $^{125}$I-KLH more slowly than normal macrophages and retained approximately three times more on their surface (Wiener & Bandieri 1975). This may explain the observed adjuvant effects on subimmunogenic antigen doses. However improved antigen presentation is unlikely to be of prime importance. This was shown by measuring the uptake of $^{51}$Cr SRBC injected at different times relative to an intravenous injection of \textit{C. parvum}. When injected 1 - 6 days after \textit{C. parvum}, splenic uptake of SRBC was reduced although by the
seventh day uptake became increased (Warr & Sljivic 1974b). Decreased splenic antigen uptake caused by oestrogens has been shown to depress antibody responses to SRBC (Warr & Sljivic 1972) whilst increased uptake by dextran sulphate enhanced antibody responses (Bradfield et al 1974); thus the depressed uptake in C. parvum treated mice might be expected to result in suppression.

Another possible way in which activated macrophages could enhance the immune response is by release of soluble factors. Factors have been isolated from cultured macrophages that enhance proliferation and differentiation of lymphocytes, although these same substances can be suppressive in high concentrations (Hoffman & Dutton 1971; Calderon et al 1975). Likewise an excess of peptone activated macrophages (more than 10:1) in cell culture usually resulted in suppression but in this system a suppressive factor was isolated (Keller 1975).

Two to four weeks after C. parvum treatment no primary anti-SRBC response could be obtained by in vitro culture of spleen cells, despite the fact that macrophages separated from these same spleens could enhance the in vitro response of normal spleen cells to SRBC (Wiener 1975). Wiener has suggested that this suppression is due to an excess of activated macrophages at this time. There is evidence that activated macrophages persist for at least 2-4 weeks after C. parvum administration. For example, C. parvum instigated trapping of lymphocytes persisted for more than 4 weeks (Frost & Lance 1973) and also protected against Brucella abortus infection for 4 weeks, although maximum protection was seen on the fourth day (Adlam et al 1972).

Antibody responses are occasionally depressed by C. parvum
treatment, particular after low antigen doses (Warr & Sljivic 1974a) and after transfer of normal spleen cells to C. parvum treated, X-irradiated recipient mice (Howard et al 1973a). These authors proposed that the enhancing effect on the antibody response, and the suppressive effect on both T-cell mediated immunity and occasionally on the antibody response, could be explained by an enhancing product from activated macrophages which suppresses at high concentrations. They suggested that T-cells have a lower threshold for suppression than do B-cells. This is supported by the observation that 2.1 mg C. parvum injected intraperitoneally suppresses both the PHA and LPS responsiveness whilst the lower dose of 0.21 mg suppresses only PHA responsiveness to the same extent but has much less effect on the LPS response (Kirchner et al 1975).

The Adjuvant Effects of B. pertussis

B. pertussis is a good adjuvant for the antibody response to SRBC when injected intravenously, intraperitoneally or subcutaneously at the same time as the antigen (Finger et al 1967; Rowley et al 1968; Dresser et al 1970b; Muro & Athanassiades 1975). A subcutaneous injection of $2.5 \times 10^8$ organisms caused lymphocyte accumulation in the draining lymph nodes (Dresser et al 1970b). This "trap" may increase the efficiency of cell recruitment and might possibly be the mechanism by which B. pertussis exerts its adjuvant effects, since it did not enhance the antibody response when injected into a site contralateral to the antigen injection (Muro & Athanassiades 1975).

When injected intraperitoneally or intravenously, B. pertussis induced profound lymphocytosis (Morse & Barron 1970; Morse & Morse 1976) which was related to alterations in lymphocyte
recirculation. Both the T and B areas of the splenic white pulp were depleted at 1-2 days, followed at 4-5 days by depletion of the lymph nodes. Lymphocytosis did not occur after subcutaneous injections (Morse 1965). A lymphocytosis promoting factor (LPP), probably analogous to the histamine sensitising factor, has been isolated and shown to be mitogenic for T-cells in vitro but this has not yet been demonstrated in vivo (Kong & Morse 1977a and b). Murgo & Athanassiades (1975) have isolated two factors from B. pertussis, one responsible for lymph node enlargement and prolongation of adjuvanticity, and the other primarily responsible for adjuvanticity. The former is a heat-labile substance, possibly LPP, the latter a heat resistant factor, possibly LPS. Early work on guinea pigs indicated that B. pertussis LPS was responsible for its adjuvanticity (Farthing 1961) whilst work on mice indicated that the heat labile component was important (Pieroni & Levine 1966 and 1967).

The importance of T-cells for B. pertussis adjuvanticity was demonstrated by both Allison and Davies (1971) and Dresser (1972). It was thought that B. pertussis exerted its adjuvant effects primarily by stimulation of T-cells, thereby increasing helper activity and antibody production. The greatest enhancement was found in the most T-dependent IgG1 class, whilst the least effect was on the IgM response (Torrigianni 1972; Dresser & Phillips 1973).

T-cells may be important for B. pertussis adjuvanticity apart from in a helper capacity, perhaps by activating macrophages. Hay and Torrigianni (1973) showed that although the IgM response to HSA was not much affected by neonatal thymectomy, thymectomy did reduce the ability of B. pertussis organisms to improve the IgM response. T-cells, primed to B. pertussis, can be stimulated by
B. pertussis in vitro to produce a factor which acts directly on the 
B-cells or macrophages (Maillard & Bloom 1972).

B. pertussis does not enhance antibody formation to the T-
independent antigens SIII and DNP-levan, and in fact has sometimes 
been shown to suppress the antibody response to SIII (Howard et al 
1973b; del Guercio & Leuchars 1972). However, at high SRBC doses 
(10^8 and 10^9) B. pertussis enhanced the response to SRBC in the 
absence of T-cells (Dresser 1972). In addition IgM and IgG 
responses to HSA were enhanced by B. pertussis in neonatally 
thymectomised mice, although not to the same extent as in normal 
mice (Hay & Torrigianni 1973). B. pertussis organisms were rapidly 
ingested by macrophages and these macrophages enhanced the in vitro 
response to SRBC whilst B. pertussis treated lymphocytes were 
ineffective (Allison 1973). If T-cells are normally required for 
macrophage activation, the enhancement of the anti-SRBC response in 
mice T-deprived/must either be attributed to stimulation of residual 
T-cells, or to direct stimulation of B-cells or macrophages. The 
inability of B. pertussis to enhance the antibody response to even 
high doses of T-independent antigens makes the direct stimulation 
of B-cells unlikely. However Unanue et al (1976) have isolated 
an enhancing factor from cultures of peptone activated macrophages 
and have shown that particle uptake by macrophages stimulated 
factor production, thus direct macrophage activation remains 
possible.

In a number of respects B. pertussis is similar in its action to 
C. parvum. Both induce excellent lymphocyte accumulation in 
draining nodes, but appear to exert their main adjuvant effects 
via activated macrophages. T-cells are required for macrophage
activation by *C. parvum* and possibly by *B. pertussis* though evidence is less extensive for this adjuvant. Both adjuvants suppress cell-mediated responses and occasionally the antibody response though again information on *B. pertussis* is limited (Eikman & Bowser 1972; Howard et al 1973b). Some differences between the two adjuvants suggest that different basic mechanisms may be acting. *C. parvum* is usually most effective when injected intravenously prior to antigen, though it does exert some adjuvant effects when injected simultaneously. *B. pertussis*, on the other hand, is more effective when injected simultaneously with antigen. In addition *C. parvum* is orientated towards both T and B cells at all SRBC doses and primarily enhances the least T-dependent IgG2a and 2b classes. *B. pertussis* is orientated towards T-cells at low SRBC doses and B-cells at high doses. It has its greatest effect on the more T-dependent IgG1 class.

**Aim of Study**

It was decided to study two main aspects of the effect of adjuvants on the response to SRBC in mice. In the first place a comparison was made of the effect of adjuvants on the numbers of PFC with their effect on the number and antibody output of B-cell clones in order to determine whether enlarged responses could be attributed to an increase in the number of clones, the number of cells per clone or the output of antibody per cell. Secondly, the development of antigen-specific B-memory cells in mice primed with adjuvant was investigated in order to discover whether the memory cell pool was expanded in parallel with the enhanced primary response. This second study was of interest since terminal exhaustion of the memory cell pool by high antigen doses in priming
has been described and attributed to the recruitment of memory cells into the primary response (Sercarz & Byers 1967; Hanna & Peters 1971). Although adjuvants are routinely used to improve priming to poorly immunogenic substances such as heterologous serum proteins, it is not known whether altered handling of an antigen such as SRBC might affect the development of memory cells within each clone.
MATERIALS AND METHODS

General

Animals, antigens and PFC assay were as described in Chapter 2. After sc injection, brachial lymph nodes were assayed for PFC, and after ip or iv immunisation spleens were assayed. IEF assay and adoptive transfers were as described in Chapter 3.

Adjuvants

1) Corynebacterium parvum CN6134 Batch P x 416 (Wellcome Research Laboratories, Beckenham).
   - sc : 175 µg C. parvum in 0.1 ml were either injected sc 4 days before sc SRBC, or were mixed with SRBC for injection at the same time.
   - ip : 700 µg C. parvum in 0.2 ml were either injected ip 4 days before ip (or iv) SRBC, or were mixed with SRBC for injection at the same time.

2) Bordetella pertussis (Wellcome Research Laboratories, Beckenham).
   - sc : 5 x 10^8 B. pertussis organisms were mixed with SRBC and injected sc in 0.1 ml.
   - ip : 2 x 10^9 B. pertussis organisms were mixed with SRBC and injected ip in 0.2 ml.
RESULTS

The Adjuvant Effects of C. parvum

C. parvum has most commonly been injected intravenously (iv), or intraperitoneally (ip) when its adjuvant action on the response to SRBC was being studied (Howard et al. 1973b; Warr & Sljivic 1974a; Warr & James 1975). The adjuvant was most effective when it was injected either ip at the same time as the antigen (Warr & James 1975) or iv a few days before antigen (Howard et al. 1973b; Warr & Sljivic 1974). It seemed of interest to study the adjuvanticity of C. parvum when injected subcutaneously (sc) and to compare it with that after ip administration. Adjuvants and antigens were always injected via the same route.

I. The Subcutaneous Route

a) The primary (1⁰) response to SRBC

In preliminary experiments 175 μg of C. parvum were injected sc into the two front footpads 4 days prior to an sc injection of 5 \( \times 10^6 \) or 5 \( \times 10^7 \) SRBC into the same two sites. The PFC responses in two such experiments are shown in Figs. 5.1 and 5.2. In the first, it is clear that C. parvum had little effect on the day 5 IgM PFC response to either 5 \( \times 10^6 \) or 5 \( \times 10^7 \) SRBC sc. The mean IgG PFC response to 5 \( \times 10^7 \) SRBC sc was increased 8 fold but this was not statistically significant due to the large variance within the control group. Fig. 5.2 shows the same lack of effect of C. parvum pretreatment on the IgG PFC response 5 days after 5 \( \times 10^6 \) SRBC sc. However, by day 7 both the IgM and IgG PFC responses were significantly higher in the C. parvum treated group, there being a 5 fold difference in IgM PFC levels and a 10 fold difference in IgG PFC levels. Thus it is clear that C. parvum pretreatment can
Fig. 5.1 Effect of C. parvum on the 10th lymph node response to SRBC

Day 5 mean log_{10} PFC response in groups of 4-5 mice injected with $5 \times 10^6$ SRBCsc(a) or $5 \times 10^7$ SRBCsc(b). A, SRBC alone; B, SRBCsc 4 days after C. parvum sc; C, C. parvum only, 9 days before assay.
Fig. 5.2 Effect of C. parvum on the \( 1^0 \) lymph node response to \( 5 \times 10^6 \) SRBC.

Day 5 and 7 PFC responses in groups of 5 mice immunised with \( 5 \times 10^6 \) SRBCsc, either alone ••••••, or 4 days after C. parvum sc ————.

I, IgM PFC

II, IgG PFC

a, responses per brachial lymph node pair

b, responses per \( 10^6 \) lymph node cells
TABLE I

**Effect of sc C. parvum and SRBC on the cellularity of the brachial lymph nodes**

<table>
<thead>
<tr>
<th>Group</th>
<th>C. parvum ( \text{(day -11)} )</th>
<th>SRBC ( \text{(day -7)} )</th>
<th>Cells/lymph node mean ( \log_{10} \pm \text{s.e.} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>-</td>
<td>-</td>
<td>1.6 ± 0.08</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>-</td>
<td>2.5 ± 0.03</td>
</tr>
<tr>
<td>C</td>
<td>-</td>
<td>+</td>
<td>*1.9 ± 0.05</td>
</tr>
<tr>
<td>D</td>
<td>+</td>
<td>+</td>
<td>2.5 ± 0.03</td>
</tr>
</tbody>
</table>

Groups of 5 mice were injected with 175μg C. parvum sc and/or 5 x 10^6 SRBC sc 11-7 days prior to assessing the cellularity of the brachial lymph nodes.

* p < 0.001 when compared to A, B and D.
prolong the late 1° PFC response. It is of interest that the enhancement is not apparent when results are expressed per 10^6 lymph node cells (Fig. 5.2b). In fact C. parvum treated groups had slightly suppressed IgM PFC responses per 10^6 Cells on both days 5 and 7, and day 5 IgG PFC were significantly suppressed. The lack of enhancement per 10^6 cells is presumably due to non-specific multiplication and recruitment of cells in the nodes in response to C. parvum (Table 1).

Day 7 and 14 sera from these experiments were analysed by IEF (Fig. 5.3). After injection of 5 x 10^6 SRBC sc, bands were apparent in the day 14 sera of control mice although few were detected in day 7 sera. In mice pretreated with C. parvum, bands were increased in number and intensity in spectra of both day 7 and 14 sera. The day 7 bleed after 5 x 10^7 SRBC sc revealed more bands than after 5 x 10^6 SRBC sc, the number and intensity of which were also increased by C. parvum pretreatment. These observations correlate well with the day 5 PFC data shown in Fig. 5.1 although there is obviously a time lag between detection of PFC and detection of antibody in the serum. The exact duration of this period is uncertain but is probably 24-48 hours since no bands could be detected in day 5 sera of mice giving good day 5 IgG PFC responses (Fig. 5.2II) but by day 7 many bands were detected (Fig. 5.4). It can be seen from the IEF spectra that C. parvum pretreatment increased the output of antibody in each clone. In another similar experiment with a strong day 7 control response to 5 x 10^6 SRBC sc, there was little difference between IEF spectra of day 7 sera from control and C. parvum treated groups but differences could be seen in a later (day 35) bleed (Fig. 5.5).
Again this suggests the *C. parvum* was sustaining the late response rather than raising the early response.

Since IEF analysis proved to be an efficient assay for adjuvant activity, this assay was used to compare the adjuvanticity of *C. parvum* when it was administered simultaneously with SRBC to that when it was administered 4 days prior to SRBC. The IEF spectra for day 7 sera raised to both $5 \times 10^6$ and $5 \times 10^7$ SRBC sc are shown in Fig. 5.6. In both instances a marked adjuvant effect was seen after pretreatment with *C. parvum* but simultaneous administration had no effect on either antigen dose.

It is clear that *C. parvum* pretreatment causes improved expression of clones recruited in response to an SRBC injection. In an attempt to determine whether clonal expansion could account for the total increase in band numbers after *C. parvum* treatment, or whether recruitment must also be affected, increasing volumes of control sera were electrofocused. The rationale was to increase the concentration of antibody from clones producing low levels, to the point where they could be detected by the IEF technique. It can be seen (Fig. 5.7A & B) that increase in clarity of faint bands was evident after a 2 fold increase in serum volume (10 µl) but further increase did not reveal more bands. Likewise an increase in volume of serum from a *C. parvum* treated mouse only intensified existing bands although a decreased volume did fail to reveal 1-2 of the weaker bands (Fig. 5.7C). These observations indicate that below a certain concentration bands cannot be detected in the gel but they are inconclusive with respect to the effect of *C. parvum* on clonal recruitment.

b) Priming for a secondary ($2^o$) response to SRBC

121.
Fig 5.3 Effect of C. parvum on the $1^0$ response to $5 \times 10^6$ and $5 \times 10^7$ SRBC

IEF spectra of sera from mice bled 7(a) and 14(b) days after immunisation with $5 \times 10^6$ (Expt. 1) or $5 \times 10^7$ (Expt. 2) SRBCsc.

A, SRBC alone; B, C. parvum 4 days before SRBCsc.
Fig 5.4  Effect of C.parvum on the 1\textsuperscript{0} response to 5\times10^{6} SRBCsc

Day 7 IEF spectra of sera from mice immunised with 5\times10^{6} SRBCsc.

A, SRBC alone; B, C.parvum 4 days before SRBCsc.
Fig. 5.5 Prolongation of the 1st response to SRBCsc by prior administration of C.parvum.

IEF analyses of day 7(1) and day 35(2) sera from mice immunised with $5 \times 10^6$ SRBCsc either alone(A), or 4 days after C.parvum sc(B).
Fig. 5.6  Comparison of adjuvant effects of C. parvum when administered at different times relative to SRBC. IEF of day 7 sera from mice immunised with 5x10^6 SRBCsc(a) or 5x10^7 SRBCsc(b). A, C. parvum sc 4 days before SRBCsc; B, SRBCsc alone; C, C. parvum sc at the same time as SRBCsc.
Fig 5.7  Effect on the IEF spectra of varying the concentration of serum applied to the gel

A and B, duplicate experiments in which 5, 10, 20, or 30 μl of day 7 sera from mice immunised with $5 \times 10^6$ SRBCsc were applied to the gel.

C, 2.5 or 10 μl of day 7 serum from a mouse injected with C.parvum 4 days before $5 \times 10^6$ SRBCsc, were applied to the gel.
Mice which had been primed with SRBC sc with or without \textit{C. parvum} 4 days previously were challenged with SRBC alone 5 weeks after priming. The 2° PFC responses can be seen in Figs. 5.8 and 5.9. Control 2° responses were extremely poor after a low dose (5 x 10^6 SRBC) challenge regardless of the priming dose (Figs. 5.8II & 5.9II). Since this dose induced a good 1° response (Fig. 5.2) the poor 2° response may be attributed to inhibition by serum antibody still present at the time of challenge (Fig. 5.5). A characteristic control 2° response was seen after challenge with 5 x 10^7 SRBC sc, with IgM and IgG PFC levels high at day 3 and falling slightly by day 5 (Figs. 5.8III & 5.9III). After priming with both 5 x 10^6 and 5 x 10^7 SRBC sc \textit{C. parvum} treated groups showed a high 2° IgG PFC response which ranged from 10 fold to 100 fold above control levels depending on the challenge dose and day of assay. A striking feature of these responses was the rise in both IgM and IgG PFC levels between days 3 and 5 for the \textit{C. parvum} treated mice. This was at a time when control 2° responses were falling and thus indicated that \textit{C. parvum} was acting to increase and sustain the late 2° PFC response in a similar manner to the 1° response.

\textit{C. parvum}, when injected four days prior to or simultaneously with a priming injection of SRBC, enhanced the 2° response to both low and high challenge doses of antigen as measured by antibody output per clone in the INF assay (Figs. 5.10 and 5.11). This was particularly marked after a high challenge dose of antigen, especially when \textit{C. parvum} was administered simultaneously (Figs. 5.10C and 5.11C).

Although clear differences were found between groups in these
experiments, it is difficult to draw conclusions in view of the results shown in Fig. 5.5 indicating that during 5 weeks of a primary response to $5 \times 10^6$ SRBC sc the IEF spectra were still strong, particularly in the sera of \textit{C. parvum} treated animals. Even if this antibody failed to suppress the $2^\circ$ response, the $2^\circ$ IEF spectra would be superimposed upon that of the $1^\circ$ and differences between control and \textit{C. parvum} treated groups would be exaggerated. $2^\circ$ IgM and IgG PFC levels were also raised when expressed per $10^6$ lymph node cells. This indicated that an adoptive transfer system might be used in order to assess the size of the memory cell pool in the absence of serum antibody. It was difficult to choose a day of assay for this type of experiment, particularly in view of the PFC results shown in Figs. 5.8 and 5.9 indicating that peak $2^\circ$ PFC response were on different days for control and \textit{C. parvum} treated mice. A day 7 assay was chosen, one day later than is usually used for adoptive $2^\circ$ responses but at a time when the adoptive $2^\circ$ response is at a plateau (Chapter 3). The disadvantage of this day of assay was that the $1^\circ$ response, particularly the IgM was high by this time (Fig. 5.12). The \textit{C. parvum} treated group, however, had a significantly raised IgG PFC response although the IgM PFC response was not significantly above control levels. This correlated with the raised IgG memory levels shown in Figs. 5.8 and 5.9. The lack of difference in the adoptive IgM response correlates with the day 3 data in these experiments, the later rise not being detected by adoptive transfer. It is difficult to determine whether this is due to removal of the primed cells from a \textit{C. parvum} environment which could potentiate the response, or to too early a day of assay.
Fig. 5.8 Effect of C. parvum on priming to $5 \times 10^6$ SRBCsc: PFC assay. Day 3 and 5 $^{20}$ PFC responses, expressed per brachial lymph node pair(a) or per $10^6$ cells(b), in groups of 5 mice primed with $5 \times 10^6$ SRBCsc and challenged 5 weeks later with $5 \times 10^6$ SRBCsc(I) or $5 \times 10^7$ SRBCsc(II). ●, primed with SRBC alone; ■, injected with C. parvum 4 days before priming with SRBCsc.

---, IgG PFC; ·····, IgM PFC.
**Fig. 5.9** Effect of *C. parvum* on priming to $5 \times 10^7$ SRBCsc:PFC assay. Day 3 and 5 $2^0$ PFC responses, expressed per brachial lymph node pair(a) or per $10^6$ cells(b), in groups of 5 mice primed with $5 \times 10^7$ SRBCsc and challenged 5 weeks later with $5 \times 10^6$(I) or $5 \times 10^7$(II) SRBCsc. •, primed with SRBC alone; ■, injected with *C. parvum* 4 days before priming with SRBCsc.

---, IgG PFC; ······, IgM PFC.
Fig 5.10  Effect of *C. parvum* on priming to $5 \times 10^6$ SRBCsc: IEF assay

IEF spectra of day 7 sera from mice primed with $5 \times 10^6$ SRBCsc and challenged 5 weeks later with $5 \times 10^6$ SRBCsc(a) or $5 \times 10^7$ SRBCsc(b). A, injected with *C. parvum* sc 4 days before priming with SRBCsc; B, primed with SRBCsc alone; C, injected with *C. parvum* sc at the time of priming with SRBCsc.
Fig. 5.11 Effect of C. parvum on priming with $5 \times 10^7$ SRBCsc: IEF assay

IEF spectra of day 7 sera from mice primed with $5 \times 10^7$ SRBCsc and challenged 5 weeks later with $5 \times 10^6$ SRBCsc(a) or $5 \times 10^7$ SRBCsc(b).

A, injected with C. parvum 4 days before priming with SRBCsc;
B, primed with SRBCsc alone; C, injected with C. parvum at the time of priming with SRBCsc.
Fig. 5.12  Effect of C. parvum on priming to $5 \times 10^6$ SRBCsc: Adoptive transfer of primed cells

Mean $\log_{10}$ PFC responses in irradiated (900 r) recipients of $10^7$ brachial lymph node cells and $10^8$ SRBCsc. A, SRBCsc alone; B, donors injected with C. parvum only, 39 days before transfer; C, donors primed with $5 \times 10^6$ SRBCsc 5 weeks before transfer; D, donors injected with C. parvum 4 days before priming with SRBCsc. 5 recipients per group.
Fig 5.13  Effect of C. parvum on priming with $5 \times 10^6$ SRBCsc: Adoptive transfer of primed cells

IEF analyses of day 7 sera from irradiated (900r) recipients of $10^7$ brachial lymph node cells and $10^8$ SRBCiv-A, donors injected with C. parvum alone, 39 days before transfer; B, donors primed with $5 \times 10^6$ SRBCsc 5 weeks before transfer; C, donors injected with C. parvum 4 days before priming with SRBCsc.
Very little difference between the groups was seen in the IEF assay (Fig. 5.13). A few more bands were present in the sera of recipients of cells from *C. parvum* treated mice but these results were inconclusive due to one strongly responding mouse in the control 2^6 (Fig. 5.13). The discrepancy in band number between individual mice in a recipient group indicates that limiting numbers of memory cells were transferred. It should be remembered that these were recipients of pooled cells from 5 donor mice, thus the sum of the bands in a recipient group indicates the number of memory precursors in the transferred cell suspensions. Assessed in this way the cell suspensions from *C. parvum* treated mice contained more memory cells than those from mice primed with SRBC alone (79 bands compared with 46 bands).

II The Intraperitoneal Route

a) The primary response to SRBC - enhancement by *C. parvum*

In accordance with the findings of Warr and James (1975), *C. parvum* raised the IgM and IgG PFC responses to SRBC when injected ip simultaneously with $5 \times 10^6$ or $5 \times 10^7$ SRBC (Fig. 5.14). The greatest enhancement followed the lower SRBC dose with the IgM response raised approximately 8 fold and the IgG response raised more than 1000 fold. Following the higher dose of $5 \times 10^7$ SRBC ip the IgM PFC response was raised 4-5 fold and the IgG response more than 10 fold. That this was not purely non-specific enhancement accounted for by the increase in splenic cell numbers induced by *C. parvum*, was demonstrated by expression of PFC numbers per $10^6$ spleen cells (Table II). In each case simultaneous administration of *C. parvum* with the SRBC increased the IgM and IgG PFC/$10^6$ cells.

The IEF spectra of these experiments are shown in Fig. 5.15.
**Fig. 5.14**  Effect of simultaneous administration of *C. parvum* on the 10\(^{th}\) response to SRBC\(_{ip}\).

Day 7 PFC responses in groups of 5 mice injected with (a) \(5 \times 10^6\) SRBC\(_{ip}\) or (b and c) \(5 \times 10^7\) SRBC\(_{ip}\).

A, SRBC\(_{ip}\) alone; B, *C. parvum* injected ip at the same time as SRBC\(_{ip}\).
### TABLE II

Enhancement of the 1° response to SRBC ip by simultaneous administration of *C. parvum*

<table>
<thead>
<tr>
<th>SRBC</th>
<th><em>C. parvum</em></th>
<th>IgM PFC/10^6 cells log_{10} mean ± s.e.</th>
<th>IgG PFC/10^6 cells log_{10} mean ± s.e.</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) 5 x 10^6</td>
<td>–</td>
<td>1.73 ± 0.25</td>
<td>0.58 ± 0.43</td>
</tr>
<tr>
<td>5 x 10^6</td>
<td>+</td>
<td><strong>2.39 ± 0.13</strong></td>
<td><strong>2.37 ± 0.27</strong></td>
</tr>
<tr>
<td>b) 5 x 10^7</td>
<td>–</td>
<td>2.96 ± 0.10</td>
<td>2.86 ± 0.08</td>
</tr>
<tr>
<td>5 x 10^7</td>
<td>+</td>
<td><strong>3.57 ± 0.05</strong></td>
<td><strong>4.03 ± 0.08</strong></td>
</tr>
<tr>
<td>c) 5 x 10^7</td>
<td>–</td>
<td>2.24 ± 0.14</td>
<td>2.13 ± 0.18</td>
</tr>
<tr>
<td>5 x 10^7</td>
<td>+</td>
<td>2.47 ± 0.07</td>
<td><strong>2.87 ± 0.11</strong></td>
</tr>
</tbody>
</table>

Groups of 5 mice were immunised with 5 x 10^6 or 5 x 10^7 SRBC ip either alone or with 700μg *C. parvum* ip. Day 7 PFC results are expressed as mean log_{10} PFC per 10^6 spleen cells.

*p < 0.05     **p < 0.01     ***p < 0.001
Fig. 5.15 **Effect of simultaneous administration of C. parvum on the 1⁰ response to SRBCip.**

IEF spectra of day 7 sera from mice immunised with 5x10⁶ SRBCip(a) or 5x10⁷ SRBCip(b). A, SRBCip alone; B, C. parvum injected ip at the same time as SRBCip.
TABLE III

Prolongation of the 1° response to SRBC ip by administration of *C. parvum*

<table>
<thead>
<tr>
<th>C. parvum</th>
<th>Day 35 PFC response</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>IgM PFC/spleen (log$_{10}$ mean ± s.e.)</td>
</tr>
<tr>
<td></td>
<td>3.12 ± 0.04</td>
</tr>
<tr>
<td>-</td>
<td>2.73 ± 0.06</td>
</tr>
</tbody>
</table>

Responses of groups of 5 mice immunised with $5 \times 10^7$ SRBC ip either alone or with 700μg *C. parvum* ip.

Results are expressed as mean log$_{10}$ PFC/spleen.
Fig. 5.16  Prolongation of the 1° response to SRBCip by administration of C.parvum.

IEF analyses of day 7(1) or day 35(2) sera from mice immunised with $5 \times 10^7$ SRBCip injected alone(A) or with C.parvum ip(B).
Mice were bled on day 7 immediately before plaqueing so individual sera can be directly related to individual PFC responses. Fig. 5.15a shows very few bands in the control group injected with $5 \times 10^6$ SRBC ip alone. This is to be expected since $5 \times 10^6$ is a low dose for ip injection. In contrast, the \textit{C. parvum} treated group have such well-developed bands that they have fused. There were more bands in the control responses to $5 \times 10^7$ SRBC ip and \textit{C. parvum} similarly expanded the number and intensity of these so that smears again developed after a 2 hour incubation with complement (Fig. 5.15b).

At day 35 of the primary response to $5 \times 10^7$ SRBC ip IgG PFC levels were significantly higher in \textit{C. parvum} immunised animals than in control groups (Table III). The IEF spectra of these mice were still clear, though not as strong as the day 7 sera, whilst no bands could be detected in control groups at this time (Fig. 5.16). It is thus clear that \textit{C. parvum} prolongs the 1\textsuperscript{st} response to ip SRBC.

b) The primary response to SRBC - suppression by \textit{C. parvum}

When \textit{C. parvum} was injected ip at the same time as $5 \times 10^6$ SRBC ip, it did not always raise the response as measured by the number of PFC and output of antibody (IEF assay). Fig. 5.17 shows two experiments in which \textit{C. parvum} treatment had no significant effect on either the IgM or IgG PFC responses to $5 \times 10^6$ SRBC ip in contrast to the marked enhancement seen in Fig. 5.14a to this same antigen dose. IEF analyses of the day 7 sera from the experiment shown in Fig. 5.16a indicated that 4/5 mice in the control group gave a reasonably heterogeneous clonal response. In contrast, 4 bands only could be detected in just 2/5 of the \textit{C. parvum} treated mice' (Fig. 5.18), and none at all when \textit{C. parvum} was administered prior to SRBC.

Suppression of the response to SRBC ip was consistently
observed when *C. parvum* was injected ip 4 days prior to injection of 5 x 10⁶ or 5 x 10⁷ SRBC ip (Fig. 5.19). The suppressive effect was greatest with the lower antigen dose, the IgM PFC response being reduced by at least 10 fold and the IgG PFC response, where present (e.g. Fig. 5.19b) being suppressed more than 100 fold. The IgM PFC response to 5 x 10⁷ SRBC ip was unaffected by prior administration of *C. parvum* although the mean level of IgG PFC was reduced almost 10 fold. This was not statistically significant due to the variation within the *C. parvum* group. Sera from these mice, when analysed by IEP showed no bands in response to 5 x 10⁶ SRBC ip (Fig. 5.20a) and bands in only one mouse in response to 5 x 10⁷ SRBC ip (Fig. 5.20b1). Another experiment shown in Fig. 5.20-b2 confirms this suppressive effect of *C. parvum* on the response to 5 x 10⁷ SRBC ip although again it is less pronounced in one of the 5 mice. This escape, or partial escape, of the occasional mouse from suppression of antibody production probably accounts for the large variability in IgG PFC responses within *C. parvum* treated groups of mice (Fig. 5.19c). Since no mice escaped *C. parvum* induced suppression of the response to 5 x 10⁶ SRBC ip, it is likely that 5 x 10⁷ is a borderline dose of SRBC for suppression.

The day 35 ¹⁰ PFC responses to 5 x 10⁷ SRBC ip were low in both control and *C. parvum* treated mice, indicating that there was little escape from suppression later in the ¹⁰ response. The day 35 IEP spectra of sera from *C. parvum* suppressed mice were interesting (Fig. 5.21). The one mouse which partially escaped from suppression early in the response showed an intense spectrum at day 35 although the spectrum was less heterogeneous than that of the day 7 controls. The other suppressed mice which had no
Fig. 5.17 Lack of effect of ip administration of C. parvum on the splenic PFC response to SRBCip.

Day 7 PFC responses of groups of 5 mice immunised with $5 \times 10^6$ SRBCip, injected either alone (A) or with C. parvum ip (B).
The diagram shows the mean log₁₀ PFC ± se for IgM and IgG in samples A and B.

- **a)**: IgM and IgG in samples A and B.
  - IgM: Sample A has a higher mean log₁₀ PFC compared to sample B.
  - IgG: Sample A has a lower mean log₁₀ PFC compared to sample B.

- **b)**: IgM and IgG in samples A and B.
  - IgM: Sample A has a higher mean log₁₀ PFC compared to sample B.
  - IgG: Sample A has a lower mean log₁₀ PFC compared to sample B.
Fig. 5.18  Suppression of antibody production by ip administration of C.parvum

IEF analyses of day 7 sera from mice immunised with $5 \times 10^6$ SRBCip.
A, C.parvum injected ip 4 days before SRBCip; B, $5 \times 10^6$ SRBC injected alone; C, C.parvum injected at the same time as SRBCip.
Fig. 5.19  Suppression of the splenic PFC response to SRBCip by prior administration of C. parvum.

Day 7 PFC responses in groups of 5 mice immunised with $5 \times 10^6$ SRBCip (a and b) or $5 \times 10^7$ SRBCip(c).

A, SRBCip alone; B, C. parvum injected ip 4 days before SRBCip.
<table>
<thead>
<tr>
<th>IgM</th>
<th>IgG</th>
</tr>
</thead>
</table>

a. $5 \times 10^6$

b. $5 \times 10^6$

c. $5 \times 10^7$

mean $\log_{10}$ PFC ± se

A  B  A  B  A  B
Fig. 5.20  Suppression of antibody production in response to ip SRBC by prior administration of C.parvum.

IEF spectra of day 7 sera from mice immunised with $5 \times 10^6$SRBCip(a) or $5 \times 10^7$SRBCip(b).

A, SRBC alone; B, C.parvum injected ip 4 days before SRBCip.
Fig. 5.21  Suppression of the late $^{10}$ response to SRBCip by *C. parvum* administered 4 days previously.

IEF spectra of day 7(1) or day 35(2) sera from mice immunised with $5 \times 10^7$ SRBCip.

A, SRBCip alone; B, *C. parvum* injected ip 4 days before SRBCip.
Fig. 5.22 Enhancement of the splenic response to intravenous SRBC by prior ip administration of C.parvum.

Day 5 and 7 PFC responses in groups of 5 mice immunised with 5x10^6 SRBCiv. •,SRBCiv alone; ■,C.parvum injected ip 4 days before SRBCiv.
Fig. 5.23 Enhancement of antibody output in response to iv SRBC by prior administration of *C. parvum*.

IEF analyses of day 7 sera from mice immunised with $5 \times 10^6$ SRBCiv.

A, SRBCiv alone; B, *C. parvum* injected ip 4 days before SRBCiv.
bands at day 7 showed a few in day 35 sera indicating expansion of one or two clones which escaped total suppression. Control groups, injected with $5 \times 10^7$ SRBC ip alone, had weak spectra by this time. It would appear that _C. parvum_ acted to expand and sustain the response of clones which escaped from its suppressive influence.

It is unlikely that ip _C. parvum_ acted by inducing production of suppressor factors since this same route and timing of injections has been used to enhance the response to $5 \times 10^6$ SRBC injected intravenously. There was more than a 10 fold increase in the IgM PFC response and more than a 5 fold increase in the IgG PFC response (Fig. 5.22). The intensity of day 7 IEF spectra was also increased (Fig. 5.23).

c) Priming for a secondary response to SRBC

Mice which had been primed with $5 \times 10^7$ SRBC ip with or without a simultaneous injection of _C. parvum_ were challenged with either $5 \times 10^6$ or $5 \times 10^7$ SRBC ip 5 weeks after priming. The day 3 and 5 $2^\circ$ PFC responses of these mice are shown in Fig. 5.24. Low dose challenge induced only a low and transient $2^\circ$ IgG PFC response (Fig. 5.24a). Challenge with the higher dose of $5 \times 10^7$ SRBC ip induced slightly higher day 3 $2^\circ$ IgG PFC which remained at the same level until day 5. Simultaneous administration of _C. parvum_ with the priming antigen injection did not affect the day 3 $2^\circ$ IgG PFC levels but raised the day 5 responses to both challenge doses. IEF analyses of day 7 sera from mice identically primed and challenged correlate well with these PFC data (Fig. 5.25). Mice injected with _C. parvum_ at the time of priming had increased antibody output per clone in the $2^\circ$ response compared with control mice injected with SRBC alone. However it is possible that the enhancement of IEF
Fig. 5.24 Effect of simultaneous administration of C. parvum on priming to SRBCip.

Day 3 and 5 $2^0$ PFC responses in groups of 5 mice primed with $5 \times 10^7$ SRBCip and challenged 5 weeks later with (a)$5 \times 10^6$ or (b)$5 \times 10^7$ SRBCip.

•, mice primed with SRBCip alone; ■, mice injected with C. parvum at the time of priming to SRBCip.

......, IgM PFC;  ———, IgG PFC.
Fig. 5.25  Effect of simultaneous administration of C. parvum on priming to SRBCip.

IEF spectra of day 7 sera from mice primed with $5 \times 10^7$ SRBCip and challenged 5 weeks later with $5 \times 10^6$ (a) or $5 \times 10^7$ (b) SRBCip.  
A, mice primed with SRBCip alone; B, mice injected with C. parvum ip at the time of priming to SRBC.
Fig. 5.26 Effect of simultaneous administration of C. parvum on priming to SRBCiP. Adoptive transfer of primed cells

Day 7 PFC responses in irradiated (900r) recipients of $10^7$ spleen cells and $10^8$ SRBCiP.

A, SRBCiP alone; B, donors injected with C. parvum ip, alone, 5 weeks before transfer; C, donors primed with $5 \times 10^7$ SRBCiP 5 weeks before transfer; D, donors injected with C. parvum at the time of priming to SRBCiP.
Fig. 5.27 Effect of simultaneous administration of *C. parvum* on priming to SRBCip: Adoptive transfer of primed cells.

IEF analyses of day 7 sera from irradiated (900r) recipients of $10^7$ spleen cells and $10^8$ SRBCiv.

A, donors injected with *C. parvum* ip 5 weeks before transfer;
B, donors primed with $5 \times 10^7$ SRBCip 5 weeks before transfer;
C, donors injected with *C. parvum* ip at the time of priming to SRBCip.
spectra was exaggerated by the residual primary antibody which was still pronounced in *C. parvum* treated groups at this time (Fig. 5.16).

An adoptive transfer experiment was therefore performed to test the effect of *C. parvum* on priming with $5 \times 10^7$ SRBC ip. Both the IgM and IgG adoptive 2° PFC responses were significantly raised above normal 2° levels (Fig. 5.26) and the antibody clones were more expanded (Fig. 5.27).

*C. parvum* administered ip 4 days prior to a priming injection of $5 \times 10^7$ SRBC ip gave unexpected results on subsequent challenge. Although 1° responses were normally suppressed by this treatment, day 3 2° IgM levels were identical in *C. parvum* treated and control groups. By day 5 after challenge, *C. parvum* treated mice gave a significantly higher IgM PFC response, indicating a potentiation of the late IgM 2° response (Fig. 5.28). 2° IgG PFC responses were high in control groups challenged with $5 \times 10^7$ SRBC ip but were poor and transient after challenge with $5 \times 10^6$ SRBC ip. 2° IgG PFC were low in both groups primed with *C. parvum* prior to SRBC. Nevertheless *C. parvum* treatment before priming considerably increased the 2° IgG antibody output as measured in the IEF assay (Fig. 5.29). This was seen best after the high dose challenge. It is unclear why there should be this discrepancy between the PFC and IEF assays. IEF analyses of day 35 1° sera showed that there was little antibody present in these mice at the time of challenge, due to suppression of the primary response.

Adoptive transfer of spleen cells from mice primed with SRBC alone, or with *C. parvum* 4 days prior to SRBC, indicated that 2° PFC responses and IEF spectra of these two groups are similar (Figs. 5.30 and 5.31). Day 7 may have been too early to detect.
**Fig. 5.28** Effect of prior administration of *C. parvum* on priming to SRBCip.

Day 3 and 5 $2^\circ$ PFC responses in groups of 5 mice primed with $5 \times 10^7$ SRBCip and challenged 5 weeks later with (a)$5 \times 10^6$ or (b)$5 \times 10^7$ SRBCip. ●, primed with SRBC alone; ■, injected with *C. parvum* ip 4 days before priming with SRBC.

........, IgM PFC; ———, IgG PFC.
Fig. 5.29 Effect of prior administration of C.parvum on priming to SRBCip.

IEF analyses of day 7 sera from mice primed with $5 \times 10^7$ SRBCip and challenged 5 weeks later with (a)$5 \times 10^6$ or (b)$5 \times 10^7$ SRBCip.

A, mice injected with C.parvum 4 days before priming with SRBC; B, mice primed with SRBCip alone.
Fig. 5.30  Effect of prior administration of C. parvum on priming to SRBCs: Adoptive transfer of primed cells.

Day 7 PFC response in irradiated (900r) recipients of $10^7$ spleen cells and $10^8$ SRBCs. A, SRBCs alone; B, donors injected with C. parvum alone, 39 days before transfer; C, donors primed with $5 \times 10^7$ SRBCs 5 weeks before transfer; D, donors injected with C. parvum 4 days before priming.
**Fig. 5.31** Effect of prior administration of *C. parvum* on priming to SRBCip: Adoptive transfer of primed cells.

IEF analyses of day 7 sera from irradiated (900r) recipients of $10^7$ spleen cells and $10^8$ SRBCiv.

A, donors injected with *C. parvum* 39 days before transfer;
B, donors primed with $5 \times 10^7$ SRBCip 5 weeks before transfer;
C, donors injected with *C. parvum* ip 4 days before priming to SRBC.
potentiation of the late $2^\circ$ response. This has already been discussed with reference to sc priming.

These results indicate that generation of memory is not hindered by suppression of the primary response since the memory cell pool size was similar to that of controls. As will be discussed later, responses seen after in situ challenge may be due to the presence or absence of feedback control by antibody in the $2^\circ$ response.

The Adjuvant Effects of B. pertussis

I  The Subcutaneous Route
a)  The primary response to SRBC

To increase the $1^\circ$ response to ip SRBC, $2 \times 10^9$ B. pertussis organisms are usually injected simultaneously and by the same route (Dresser et al 1970b). As with C. parvum, the dose was reduced for sc injection in these experiments to a quarter of that given ip. The adjuvant effect of $5 \times 10^8$ B. pertussis organisms on the primary response to sc injections of $5 \times 10^6$ and $5 \times 10^7$ SRBC is shown in Fig. 5.32. The IgM PFC response to $5 \times 10^6$ SRBC sc was raised 5 fold, and the IgG response (where present) was similarly increased. The increase was still apparent when PFC results were expressed per $10^6$ lymph node cells. Neither the IgM nor IgG PFC responses to $5 \times 10^7$ SRBC sc were significantly raised by administration of B. pertussis. The IEF spectra of day 7 sera from mice injected with $5 \times 10^6$ or $5 \times 10^7$ SRBC sc are shown in Figs. 5.33 and 5.34. B. pertussis treatment had no effect on the day 7 spectra. However IEF analyses of day 14 sera raised to $5 \times 10^6$ SRBC sc showed intensified bands in sera from B. pertussis treated mice indicating a limited potentiation of the late $1^\circ$ response.

129.
Fig. 5.32  Effect of B. pertussis on the $1^{0}$ response to SRBCs.

a and b, two experiments showing day 5 PFC responses of groups of 5 mice immunised with $5 \times 10^{6}$ SRBCs (A and F); $5 \times 10^{6}$ SRBC + $5 \times 10^{8}$ B. pertussis sc (B, G and H); $5 \times 10^{7}$ SRBCs (C); $5 \times 10^{7}$ SRBC + $5 \times 10^{8}$ B. pertussis sc (D); or unimmunised (E).

* a different batch of B. pertussis was used in group H.
Fig. 5.33  Effect of B. pertussis on the 10 response to SRBCsc.

IEF spectra of sera from mice bled 7(1) or 14(2) days after immunisation. A, 5x10^6 SRBCsc alone; B, 5x10^6 SRBCsc + 5x10^6 B. pertussis sc.
Fig. 5.34 Effect of *B. pertussis* on the 1⁰ response to SRBCsc.

Duplicate experiments showing IEF spectra of day 7 sera from mice immunised with A, $5 \times 10^7$ SRBCsc alone; B, $5 \times 10^7$ SRBCsc + $5 \times 10^8$ *B. pertussis*.
Fig. 5.35  Effect of B. pertussis on priming to $5 \times 10^6$ SRBCsc.

Day 3 and 5 PFC responses in groups of 5 mice primed with $5 \times 10^6$ SRBCsc and challenged 5 weeks later with (a)$5 \times 10^6$ or (b)$5 \times 10^7$ SRBCsc.

■, primed with SRBCsc alone; ○, primed with SRBCsc + $5 \times 10^8$ B. pertussis.

-----, IgM PFC; -----, IgG PFC.
Fig. 5.36  Effect of B.pertussis on priming to $5 \times 10^7$ SRBCsc.

Day 3 and 5 PFC responses in groups of 5 mice primed with $5 \times 10^7$ SRBCsc and challenged 5 weeks later with (a)$5 \times 10^6$ or (b)$5 \times 10^7$ SRBCsc.

■, primed with SRBC alone; ●, primed with SRBC + $5 \times 10^8$ B.pertussis.

........ IgM PFC; ----- , IgG PFC.
Fig. 5.37 Effect of *B. pertussis* on priming to SRBCsc.

IEF spectra of day 7 sera from mice primed with $5 \times 10^6$ SRBCsc and challenged 5 weeks later with (a)$5 \times 10^6$ or (b)$5 \times 10^7$ SRBCsc.

A, primed with SRBCsc alone; B, primed with SRBCsc + $5 \times 10^8$ *B. pertussis*. 
Fig. 5.38 Effect of B. pertussis on priming to SRBCsc

ISF spectra of day 7 sera from mice primed with $5 \times 10^7$ SRBCsc and challenged 5 weeks later with (a)$5 \times 10^6$ or (b)$5 \times 10^7$ SRBCsc.

A, primed with SRBC alone; B, primed with SRBC + $5 \times 10^3$ B. pertussis.
(Fig. 5.33). A later (day 7) PFC assay may have more clearly revealed differences between control and *B. pertussis* treated mice since it is likely that increased PFC numbers per clone are responsible for the intensified IEF spectra.

b) Priming for a secondary response to SRBC

Experimental mice were injected with *B. pertussis* incorporated with a priming injection of $5 \times 10^6$ or $5 \times 10^7$ SRBC sc. 5 weeks later these mice were challenged with either the low ($5 \times 10^6$) or high ($5 \times 10^7$) antigen dose. $2^\circ$ PFC results are shown in Figs. 5.35 and 5.36. There was no significant difference in $2^\circ$ IgM PFC levels between *B. pertussis* treated and control groups for either high or low priming and challenge injections. However day $5^\circ$ IgG PFC levels were significantly raised above control $2^\circ$ levels in mice primed with $5 \times 10^6$ SRBC sc plus *B. pertussis*. After a high dose challenge IgG PFC levels were significantly raised by day 3. Significantly higher $2^\circ$ IgG levels were also seen after high dose priming and challenge but were not apparent after high dose priming and low dose challenge. IEF spectra of day 7 sera from identically treated mice are shown in Figs. 5.37 and 5.38. Enhancement of $2^\circ$ antibody production (as detected by this assay) was most pronounced in *B. pertussis* treated groups after challenge with $5 \times 10^7$ SRBC sc (Figs. 5.37b and 5.38b) and particularly so after priming with the lower SRBC dose. There was not much difference between control and *B. pertussis* treated groups after $5 \times 10^6$ SRBC sc challenge regardless of the priming antigen dose.

II The Intraperitoneal Route

a) The primary response to SRBC

$2 \times 10^9$ *B. pertussis* organisms injected ip at the same time as
SRBC ip, had no effect on the day 7 IgM and IgG PFC responses to $5 \times 10^6$ SRBC ip but significantly raised the IgM and IgG PFC responses to $5 \times 10^7$ SRBC ip by approximately 4 fold (Fig. 5.39). The IEF spectra of day 7 sera from two similar experiments are shown in Fig. 5.40. \textit{B. pertussis} was found to increase the antibody content per clone, to both doses of SRBC. The differences between results of these two assays probably reflect variability between experiments in the kinetics of the ip response to SRBC.

b) \textbf{Priming for a secondary response to SRBC}

$2 \times 10^9$ \textit{B. pertussis} organisms, injected with a priming injection of $5 \times 10^7$ SRBC ip, did not increase the $2^\circ$ IgM PFC response after a 5 week challenge (Fig. 5.41). The $2^\circ$ IgG response was raised in mice treated with \textit{B. pertussis} at the time of priming. This was only statistically significant after the low dose challenge. IEF analyses of day 7 sera after challenge showed enhancement of the clonal spectra as a consequence of \textit{B. pertussis} injection with the priming antigen dose. This enhancement was evident for both priming antigen doses (Figs. 5.42 and 5.43).
Fig. 5.39  Effect of B. pertussis on the 1\textsuperscript{st} response to SRBC\textsuperscript{ip}.

Day 7 PFC responses of groups of 5 mice immunised with:

A, 5x10\textsuperscript{6} SRBC\textsuperscript{ip}; B, 5x10\textsuperscript{6} SRBC + 2x10\textsuperscript{9} B. pertussis ip; C, 5x10\textsuperscript{7} SRBC\textsuperscript{ip};
D, 5x10\textsuperscript{7} SRBC + 2x10\textsuperscript{9} B. pertussis ip.
Fig. 5.40 Effect of B. pertussis on the 1\textsuperscript{st} response to SRBCip.

IEF spectra of day 7 sera from mice immunised with 5x10\textsuperscript{6} SRBCip(1) or 5x10\textsuperscript{7} SRBCip(2). A, SRBCip alone; B, SRBC injected ip with 2x10\textsuperscript{9} B. pertussis.
Fig. 5.41 Effect of B.pertussis on priming to SRBCip.

Day 3 and 5 20 PFC responses in groups of 5 mice primed with 5\times10^7 SRBCip and challenged 5 weeks later with (a)5\times10^6 or (b)5\times10^7 SRBCip.

- •, primed with SRBCip alone; ■ ●, primed with 2\times10^9 B.pertussis + SRBCip.

......, IgM PFC; ——, IgG PFC.
**Fig. 5.42** Effect of *B. pertussis* on priming to SRBCip.

IEF spectra of day 7 sera from mice primed with $5 \times 10^6$ SRBCip and challenged 5 weeks later with (a) $5 \times 10^6$ or (b) $5 \times 10^7$ SRBCip.

A, primed with SRBCip alone; B, primed with SRBC + $2 \times 10^9$ *B. pertussis* ip.
Fig. 5.43  Effect of B. pertussis on priming to SRBCip.

IEF spectra of day 7 sera from mice primed with 5x10^7 SRBCip and challenged 5 weeks later with (a)5x10^6 or (b)5x10^7 SRBCip. 

A, primed with SRBCip alone; B, primed with SRBC + 2x10^9 B. pertussis ip.
DISCUSSION

The Enhancing Effects of C. parvum

a) The primary response to SRBC

The effects of C. parvum on the humoral antibody response to SRBC have previously been studied following intravenous or intraperitoneal administration of adjuvant (Howard et al. 1973b; Warr & Sljivic 1974a; Warr & James 1975). The experiments presented in this Chapter showed that it can also act as a potent adjuvant when injected subcutaneously 4 days prior to a subcutaneous injection of SRBC. Enhancement was primarily directed at the late primary response since differences in IgM and IgG PFC levels between C. parvum treated and control mice were first seen in a day 7 assay (Figs. 5.1 and 2). This day 7 difference could be attributed both to a fall in control responses from peak levels at day 5 and to a continued rise in C. parvum treated animals between days 5 and 7. The enhancement of PFC responses was reflected in the increased antibody per clone as measured by IEF analysis of day 7 or 14 sera raised to 5 x 10^6 or 5 x 10^7 SRBC sc (Figs. 5.3, 4, 5 and 6). The implications of this in terms of clonal recruitment and expansion will be considered in more detail later. When C. parvum was injected sc simultaneously with antigen it did not enhance the day 7 IEF spectra to either SRBC dose (Fig. 5.6). Later sera may have shown differences but it is clear that prior administration of C. parvum by the sc route results in a more effective enhancement. This was also true for the intravenous route (Howard et al. 1973b; Warr & Sljivic 1974a). However Warr and James (1975) showed that C. parvum injected intra-peritoneally was more effective at enhancing IgG PFC responses when given simultaneously with SRBC rather than 7 days previously.
The adjuvant effect of \textit{C. parvum} injected ip at the same time as SRBC ip has been confirmed in the experiments presented in this Chapter, and was found to be true for immunisation with both $5 \times 10^6$ and $5 \times 10^7$ SRBC (Fig. 5.14). The degree of PFC enhancement on day 7 was comparable to that seen after sc administration. IgM PFC were raised approximately 5 fold and IgG PFC more than 10 fold, the greater effect being seen on the lower antigen dose. This was clearly reflected in the day 7 IEF spectra indicating that there was an increase in antibody output per clone. Warr and James (1975) showed enhancement of IgM and IgG PFC levels to doses of SRBC ranging from $3 \times 10^6$ – $3 \times 10^9$. They found a 3-4 fold enhancement for IgM, IgG2a and IgG2b but increases in IgG2a and 2b PFC were most pronounced at the lower doses of $3 \times 10^6$ and $3 \times 10^7$ SRBC. The IgG1 PFC were least affected, seldom being significantly enhanced and sometimes slightly suppressed. No IgG class distinction has been made in experiments presented here but the differential effects on IgG classes should be remembered.

Enhancement via the intraperitoneal route was very apparent whether PFC numbers were expressed per spleen or per $10^6$ spleen cells. This suggests that despite the non-specific increase in spleen cell number induced by \textit{C. parvum}, antigen-specific cells were preferentially induced to accumulate when adjuvant and antigen were injected ip together (Table II). On the other hand, sc \textit{C. parvum} did not increase the number of anti-SRBC PFC per $10^6$ lymph node cells despite the increase per lymph node. The depressed day 3 PFC response per $10^6$ lymph node cells in \textit{C. parvum} treated groups probably reflects the time lag required for PFC to develop (Fig. 5.2). Between days 3 and 5 PFC numbers increased in proportion to the total cell numbers,
presumably by preferential adjuvant action on these cells and/or their precursors.

A more sustained response to SRBC than normal was obtained after *C. parvum* treatment for both the sc and ip routes and this was clearly demonstrated in both PFC and IIF assays 35 days after immunisation (Fig.5.2 and Table II; Figs.5.5 and 16). This potentiation of the late PFC response may be explained by an increase in the lifespan of individual AFC and/or a resistance to feedback signals allowing continued division of cells, presumably in response to persistent antigen in the *C. parvum* treated group. In order to explain the continued increase in IgG PFC between days 5 and 7 of the sc response, the latter must be true. The former may or may not occur. Resistance to feedback inhibition may be the result of an increase in the proportion of helper T-cells relative to suppressor T-cells. In a normal immune response both helper and suppressor cells are generated and the response is a reflection of the balance between the two effects (Chan & Henry 1976). Peptone-activated macrophages produce a factor which increases helper activities (Calderon et al 1975) thus it would not be difficult to imagine a system in which T-helper cells were preferentially increased by *C. parvum* treatment. This type of effect might be expected to preferentially enhance the more helper T-dependent IgG1 class. Certainly IgG responses were enhanced more than IgM (Figs.5.2 and 14), but evidence from Warr and James (1975) indicated that this mainly affected the least T-dependent IgG2a and 2b classes.

Suppressor T-cells may exert their effects by inducing macrophages to produce a suppressor factor (Easten et al 1975).
Con A activated splenic T-cells produce a soluble immune response suppressor molecule (SIRS) which non-specifically suppresses PFC responses. The macrophage was shown to be the target cell for this suppressor molecule (Tadakuma & Pierce 1976). It is possible that *C. parvum* activated macrophages become resistant to activation by T-suppressor factors with the result that the immune response to an antigen escapes control.

Individual bands in the IEF spectra of sera from *C. parvum* treated mice were very much more intense than those from mice immunised with SRBC alone indicating that there was a much greater concentration of antibody per band. It follows from this that there was an increased output of antibody per clone. This increase may be accounted for solely by an increase in PFC numbers per clone but it is possible that, in addition, some individual cells were making more antibody. Support for this comes from the observation that more large plaques were detected in cell suspensions from *C. parvum* treated mice although plaque size was not routinely measured. However increases in plaque size may also reflect recruitment of low affinity clones or recruitment of high-rate antibody producing cells. There is some indication that adjuvants act to increase the affinity of a response (Petty & Steward 1977) thus preferential expansion of low affinity clones is unlikely. There is also evidence that Wax D from *M. tuberculosis* preferentially favours production of precursors of high-rate antibody producing cells (Tanaka et al 1975). Selection of such cells would most probably lead to accumulation of a memory cell pool of potential high-rate antibody producers. No difference was observed in secondary plaque size in the present experiments between control mice and those treated.
with \textit{C. parvum} at the time of priming, so this is unlikely to be the method of action.

It is possible that in addition to expanding existing clones, the adjuvant was also recruiting new clones. This was unlikely due to the lack of effect of so \textit{C. parvum} on the early kinetics of the PFC response to SRBC so. However the higher peak splenic response of \textit{C. parvum} treated mice (day 7 was the control peak) plus the identical early kinetics of these and control mice suggests that extra clonal recruitment may be important in \textit{C. parvum} action ip. It was hoped that the IEF analysis of sera might provide some insight into the adjuvant action on clonal recruitment and expansion but it has not proved possible to draw rigid conclusions about recruitment using this system. More bands were frequently seen in sera from \textit{C. parvum} treated groups but this might well be due to expansion of normally recruited, poorly expanded clones which the assay was not sufficiently sensitive to detect. Application of larger volumes of control sera, or smaller volumes of \textit{C. parvum} treated sera indicated that below a certain concentration of antibody, bands may be lost (Fig. 5). A two fold increase in the volume of the control serum applied to the gel increased the number of bands detected but a further 3 fold increase had no effect.

It is obvious that the main effect of \textit{C. parvum} is on clonal expansion since individual bands were always broader and more intense in sera from adjuvant treated mice and PFC numbers were raised. If clonal recruitment is also influenced it must be a relatively minor effect in that \textit{C. parvum} treated animals at most showed only slight increases in the number of detectable clonotypes. Civin \textit{et al} (1976) have recently approached this problem of clonal
expansion and/or recruitment by studying the effect of increasing antigen concentration and of Freund's-type adjuvants on the restricted clonal response to DNP-oligolysines. They showed marked clonal expansion after both treatments but were not able to detect new clones. Their work thus supports the notion that a number of adjuvants exert their effect primarily by increasing proliferation of AFC precursors. However this work could also be explained by an effect on differentiation of cells, perhaps resulting in more rapid antibody production per cell.

b) Priming for a secondary response to SRBC

It has often been reported that a large primary dose of antigen primes poorly for a secondary response to that antigen (Byers & Sercarz 1968; Hanna & Peters 1971; Grantham & Fitch 1975). It has been suggested that this low $2^\circ$ response results from depletion of the memory cell compartment by incorporation of memory cells into the primary response. This was shown in an in vivo cell transfer system by Hanna and Peters (1971). However Grantham and Fitch (1975) could not show this in an in vitro transfer system. They found that whilst a day 30 in vivo challenge induced a higher $2^\circ$ response following priming with $1 \times 10^6$ SRBC iv, a day 30 in vitro challenge induced a higher $2^\circ$ response following the higher priming dose. They, and others (Safford & Tokuda 1971) showed that passive antibody could mimic this suppression of the secondary response. Addition of 0.1 ml of undiluted anti-SRBC serum (1:512) at the time of priming did not prevent development of the memory cell pool but prevented its full expression upon challenge for at least 8 weeks after priming (Safford & Tokuda 1971). These low $2^\circ$ responses could not be imitated by priming with lower antigen
doses indicating that enhanced antigen-clearance was not the sole mechanism of antibody-mediated suppression. Such suppression may act by a direct feedback control of antibody production or, alternatively, by antibody-antigen complexes activating suppressor T-cells (Basten et al. 1975) which may then act directly, or through macrophages, to suppress the 2° response.

It is clear from the subcutaneous and intraperitoneal priming experiments in this Chapter that the memory cell pool was not exhausted during high primary responses which resulted from adjuvant administration. Neither was expression of these memory cells prevented by the high levels of serum antibody present at the time of challenge. These points were particularly well-illustrated after sc injections when primary responses were potentiated with strong IEF spectra still evident in C. parvum treated groups at day 35 (Fig.55). Challenge at this time with high or low antigen doses (5 x 10⁷ or 5 x 10⁶ SRBC sc) induced high 2° IgG and IgM PFC responses and intense IEF spectra. The 2° responses of control mice, on the other hand, were best after a high challenge, perhaps because this dose most effectively overcame the feedback effects of passive antibody. Transfer of 10⁷ primed cells into X-irradiated recipients, with subsequent antigen challenge showed higher 2° IgM and IgG PFC responses and expanded IEF spectra in recipients of cell suspensions from C. parvum treated mice though the difference between these and controls was not as pronounced as after in vivo challenge. It is clear that treatment of mice with C. parvum sc 4 days prior to a priming injection of SRBC sc expanded the anti-SRBC memory cell pool of these animals. Adoptive transfer of cells after ip priming indicates that the same memory pool expansion occurs
after ip administration of *C. parvum*. Warr and Sljivic (1974a) showed that the anti-SRBC memory cell pool was increased in size by intravenous administration of *C. parvum* but they found only a 2-3 fold increase which was not significant when expressed per $10^6$ cells.

The experiments involving direct in vivo challenge of primed animals indicated that feedback control mechanisms for the $2^0$ response were abrogated by priming with adjuvant (Figs. 5, 8 and 9). Two mechanisms were considered to account for abrogation of control in the $1^0$ response and both of these could apply in the $2^0$ also. In the first place, macrophages activated by *C. parvum* may have become resistant to activation by suppressor T-cell products, for production of suppressor factors. There is evidence that *C. parvum* activated macrophages are present in the spleen for at least 4 weeks after injection of *C. parvum* iv. Secondly, *C. parvum* treatment may have biased priming in favour of large numbers of T-helper cells by which the $2^0$ response was augmented despite the activity of normal control mechanisms. Again a preferential effect on the more T-dependent antibody classes might be expected instead of the pronounced enhancement of $2^0$ IgM PFC. However Finger et al (1977) showed a similar potentiation of $2^0$ IgM PFC to SRBC after incorporating *B. pertussis* with the priming injection of SRBC. *B. pertussis* would be expected to exert its adjuvant effects primarily by augmenting T-cell help. No such augmentation of the $2^0$ IgM PFC response was seen in these experiments (see below).

c) Conclusions - site of action of *C. parvum*

*C. parvum* was able to cause a large expansion of $1^0$ B lymphocyte clones to SRBC after either an sc injection 4 days prior to sc antigen or an ip injection simultaneously with antigen. There was
little evidence for recruitment of new clones by *C. parvum* so it is likely that its main effect was to increase proliferation and differentiation within a clone. In the context of the X-Y-Z scheme of immunocyte maturation (Sercarz & Byers 1967) *C. parvum* may act at the X → Y, Y → Y or Y → Z stages of the immune response. Since the memory cell pool was expanded by the same injection protocol some of its effect at least must be at the X → Y or Y → Y stage. Massive expansion of the Y cell compartment with some of these memory cells then recruited into the 1° response would be sufficient to explain both the enhanced 1° and 2° responses but does not eliminate the possibility of additional enhanced proliferation of the Y → Z stage.

The Suppressive Effects of *C. parvum*

a) The primary response to SRBC

Intravenous administration of *C. parvum* results in suppression of T-cell mediated responses (Howard *et al* 1973b; Toujas *et al* 1973) and under certain conditions has also been reported to suppress the IgM PFC response to SRBC (Warr & Sijovic 1974a). The importance of adjuvant dose, antigen dose, route and timing of administration, in determining the influence of adjuvants is becoming increasingly apparent. Suppression of the ip response to SRBC was routinely observed in these experiments when mice were administered with *C. parvum* ip 4 days prior to antigen injection. However Warr and James (1975), also using CBA mice, found no suppression of the ip response to SRBC after prior administration of *C. parvum*. These experiments differ from mine in 3 ways:

1) the adjuvant dose was higher (1.4 mg compared with 0.7 mg);
2) the adjuvant was administered 3 days before antigen instead
of 4;

3) the dose of SRBC was higher ($3 \times 10^8$ compared with $5 \times 10^6$ and $5 \times 10^7$).

The antigen dose is probably important since in these experiments suppression was most pronounced on the lower dose of $5 \times 10^6$ SRBC ip. After $5 \times 10^7$ SRBC ip, the occasional mouse partially escaped suppression (e.g. Fig. 5.20). Clones which escaped suppression were enhanced by the adjuvant action of C. parvum since the IEF spectra for these mice showed increased antibody in those clones which appeared and were also clearly detected at day 35 when control spectra were poor (Fig. 5.21). This does not represent total escape from suppression of all competent cells late in the response since the clonal spectrum at day 35 was still restricted. Since suppression of a dose of $5 \times 10^7$ SRBC ip is less effective than that of a lower dose, it would not be surprising to find no suppression at all of a higher dose, such as the $3 \times 10^8$ SRBC ip injected by Warr and James (1975).

Suppression may be the end-result of extremely efficient phagocytosis by overactivated macrophages. Perkins (1969) showed that the magnitude of an immune response in the spleen was dependent on the amount of antigen reaching it, and that very little SRBC antigen localised in the spleen after intraperitoneal injection due to a scavenger effect of peritoneal macrophages. This scavenger effect was increased by prior peptone activation of peritoneal macrophages and reduced following their blockade by particle uptake. It was also most pronounced on doses of $2 \times 10^6$ and $2 \times 10^7$ SRBC ip but had little effect on $2 \times 10^8$ SRBC ip. There is some evidence for suppression by peptone-activated
macrophages when cultured in high concentrations relative to the number of lymphocytes (Keller 1975). Though suppressor factors have been isolated from peptone-activated macrophages it is unlikely that such factors are operating in the C. parvum system since the response to intravenous SRBC was not suppressed. Howard et al (1973b) suggested that enhancement and suppression were both mediated by the same macrophage factor. Calderon et al (1975) demonstrated synergism between the enhancing factor isolated from peptone-activated macrophages and low levels of T-helper activity. However when T-cell help was high, antagonism was demonstrated between the two.

T-cells may be more susceptible to suppression by high concentrations of macrophage factor than are B-cells (Kirchner et al 1975). Since the response to $5 \times 10^6$ SRBC is more T-dependent than that to $5 \times 10^7$ SRBC (Dresser 1972) this could feasibly explain the more pronounced suppression of the lower antigen dose. It should be remembered that enhancement, where seen, was also more pronounced on the lower antigen dose thus a delicate balance must exist between suppression and enhancement.

Splenic macrophages have been shown to suppress more effectively than peritoneal exudate macrophages and it was suggested that intravenous C. parvum activates those in the spleen (Oppenheim & Rosenstreich 1976). However in these experiments suppression was only seen after ip C. parvum which is unlikely to primarily stimulate splenic macrophages. Preliminary experiments (unpublished) have indicated that enhancement of the SRBC response occurs after both simultaneous and prior administration of C. parvum by the intravenous route.
b) Priming for a secondary response to SRBC

Despite the pronounced suppression of the 1\textsuperscript{st} PFC response, a capacity to give a good secondary response developed following priming with \textit{C. parvum} ip 4 days prior to $5 \times 10^7$ SRBC ip. This was apparent in the IgM 2\textsuperscript{nd} PFC response but not in the IgG PFC response detected in that particular assay (Fig. 5.28). On the other hand, the IEF responses of identically treated animals bled 7 days after challenge were particularly well-enhanced indicating that good IgG memory had developed (Fig. 5.29). A high challenge dose induced a diverse secondary clonal spectrum though that after low challenge was more restricted. This secondary response cannot simply reflect memory within the few clones which escaped suppression since clones escaping suppression in the 1\textsuperscript{st} response to $5 \times 10^7$ SRBC ip were restricted in number despite the fact that individual clones became expanded by \textit{C. parvum} adjuvanticity.

The IEF spectra of 2\textsuperscript{nd} responses in \textit{C. parvum} primed animals were highly heterogeneous indicating that memory developed in clones suppressed for a 1\textsuperscript{st} response.

The adoptive transfer experiment showed that there was no difference between 2\textsuperscript{nd} responses of \textit{C. parvum} treated mice and controls, when measured after 7 days by PFC or IEF assays (Figs. 5.31 and 32). Thus although memory development was not suppressed, neither was it enhanced by \textit{C. parvum} despite its effect on escaping clones. The differences seen after \textit{in vivo} challenge may have been emphasised by suppression of the control 2\textsuperscript{nd} responses by feedback antibody.

Klaus and Wilcox (1975) demonstrated a similar phenomenon when studying suppression of the primary response, and priming, in mice made tolerant to DNP by DNP-SII. A few clones escaped suppression
in the 1^o response but clonal memory, though slightly reduced, was highly heterogeneous. DNP-SIII blocks the B-cell receptor thereby preventing T-B cell interactions. They believe that B-cell priming was relatively unaffected by tolerance induction due to its lesser dependence on T-cell help. This certainly raises the possibility that macrophage factors, operating in a similar manner, could prevent T-B interactions in high concentrations whilst enhancing them at low concentrations.

c) Conclusions - site of action of C. parvum

It remains possible that the C. parvum stimulus for proliferation operates at both X→Y and Y→Z stages. No enhancement of X→Y proliferation and abrogation of Y→Z could explain these results since the memory cell pool is unaffected by prior ip administration of C. parvum. Enhancement of escaping clones indicates adjuvant activity at the Y→Z stage after escape has occurred. Reasons for lack of enhancement of the X→Y and Y→Y stages and abrogation of the Y→Z stage by this immunisation protocol, remain obscure.

The Adjuvant Effects of B. pertussis

a) The primary response to SRBC

The IgM and IgG PFC responses to 5 × 10^6 SRBC sc were both raised at least 5 fold by a simultaneous sc injection of 5 × 10^8 B. pertussis organisms. There was little effect on the 1^o IgM PFC to the higher antigen dose (5 × 10^7 SRBC sc) and the effect on the 1^o IgG PFC to this dose could not be assessed due to the variability in the control group. Different batches of B. pertussis had different degrees of effect (Fig. 5.32). Enhancement was also apparent when results were expressed per 10^6 lymph node cells indicating that B. pertussis induces an increase in the proportion of antigen-specific cells in
the lymph nodes. In fact, lymph node enlargement is not a prerequisite for adjuvanticity (Murgo & Athanassiades 1975). Murgo and Athanassiades (1975) recently showed that B. pertussis injected sc with SRBC exerted its greatest adjuvant effects on low SRBC doses \((2 \times 10^6)\) and had much less effect, or none at all, on higher doses \((2 \times 10^7\) and \(2 \times 10^8\)). They found an approximately 10 fold increase in IgM and IgG PFC responses upon administration of B. pertussis, although control IgG responses were very low. They also showed in a dose response curve for the adjuvant that \(1.5 \times 10^9\) B. pertussis organisms injected sc gave the most enhancement whilst \(3 \times 10^8\) organisms or less, had little adjuvant effect. It may be that in the present experiments the dose of B. pertussis used was rather low and was only just effective as an adjuvant although this would require testing for the batch of B. pertussis used. Mouse strain may also play a role.

The IgM PFC peaks of control and B. pertussis treated animals were on the same day and PFC responses fell at the same rate (Murgo & Athanassiades 1975). However the IgG PFC responses of B. pertussis treated animals peaked later than controls. This correlates well with the IEF data presented here which showed differences in day 14 but not in day 7 serum samples, indicating that by day 14 IgG PFC may have multiplied sufficiently to have produced more antibody per clone.

Prior to their work (Murgo & Athanassiades 1975), and that presented here, B. pertussis has usually been injected iv or ip with SRBC. This induces a profound lymphocytosis (Morse & Barron 1970) which may confuse interpretation of adjuvant effects. When injected ip B. pertussis enhanced the splenic response to optimal or maximal
SRBC doses (Dresser 1972). Once again a higher dose of $10^{10}$ organisms was more effective than $2 \times 10^9$. $2 \times 10^9$ *B. pertussis* organisms were used in these experiments for ip injection. The adjuvant effect was most pronounced on the higher antigen dose ($5 \times 10^7$ SRBC ip) although antibody output after the lower dose was also enhanced according to the IEF assay. There is some evidence that PFC peaks are later to low antigen doses (Dresser et al. 1970b) so a day 5 PFC assay was probably too early for good IgG responses to $5 \times 10^6$ SRBC ip. IgM PFC in *B. pertussis* treated mice peaked on the same day as those in control mice but the response was more sustained than in controls (Dresser et al. 1970b). IgG2a PFC peaked on the same day but the peak responses of *B. pertussis* treated mice were higher than those of controls. This indicates that *B. pertussis* may act to increase the rate of cell division (Dresser & Phillips 1973; Finger et al. 1977) or perhaps increase clonal recruitment when administered ip. There is considerable evidence that *B. pertussis* exerts its adjuvant effect primarily on the IgG1 class, IgM being the least affected (Dresser & Phillips 1973). However there was no evidence for this from these experiments in which IgM and IgG PFC were equally enhanced (Fig.5).39.

b) Priming for a secondary response to SRBC

Finger et al. (1977) studied the effects of *B. pertussis* administered ip on ip priming for a $2^\circ$ SRBC response. They found that the peak $2^\circ$ PFC response was slightly higher than that of untreated mice and that the $2^\circ$ response (particularly IgM) was more sustained. The higher peak $2^\circ$ PFC responses probably reflect an increase in the memory pool size although their challenge was at day 28 when circulating antibody may still have been high and exerting
feedback control on responses of animals not primed with adjuvant.

In the experiments recorded here there was neither an increase in IgM memory pool size nor a sustained IgM memory response after sc or ip priming with *B. pertussis*. If, as suggested for *C. parvum*, the sustained $2^\circ$ IgM response is dependent on the presence of activated macrophages which are resistant to activation by suppressor T-cells factors then it would appear that they do not remain active for the 5 week interval between priming and challenge used in these experiments. Finger et al (1977) used a shorter interval (4 weeks) and a higher dose of adjuvant ($3 \times 10^9$ *B. pertussis* organisms).

$2^\circ$ IgG PFC levels and antibody output were raised after sc administration of *B. pertussis* with both $5 \times 10^6$ and $5 \times 10^7$ SRBC sc. This was probably also true after ip priming though data were less conclusive. It would seem that the IgG memory cell pool is increased in size by *B. pertussis* administered with the priming injection of antigen.

c) Conclusion - site of action of *B. pertussis*

$1^\circ$ IgM and IgG PFC responses to SRBC were equally raised by *B. pertussis* treatment indicating enhancement of B-cell proliferation at either the $X\rightarrow Y$, $Y\rightarrow Y$ or $Y\rightarrow Z$ stages of the response. $2^\circ$ IgG levels were also raised indicating an increase in the size of the IgG memory cell pool presumably by proliferation at $X\rightarrow Y$ or $Y\rightarrow Y$. Since the IgM memory pool was not altered it seems likely that *B. pertussis* can only act on the $Y\rightarrow Z$ stage of IgM B-cell development.

There were certain similarities in the adjuvant action of *B. pertussis* and *C. parvum* in that both increased $1^\circ$ IgM and IgG
PFC levels after either sc or ip injections, in each case resulting in an increased antibody output per clone. There were however certain differences between the two in the immunisation schedules required for best enhancement, the antigen doses most enhanced and the intraperitoneal suppression by C. parvum. The adjuvant effects of sc B. pertussis could be seen in a day 5 PFC assay but those of C. parvum were not evident until day 7.

Both adjuvants increased the size of the IgG memory cell pool to SRBC when administered with the priming antigen injection. In addition, C. parvum priming raised the level of 2° IgM PFC, primarily by prolongation of the late response but also by an increase in IgM memory cell numbers as shown in an adoptive transfer. B. pertussis, on the other hand, had little effect on the 2° IgM response. Differences may have been shown up by an adoptive transfer.
CHAPTER 6

LYMPHOCYTE RECIRCULATION: THE DISTRIBUTION OF MEMORY

CELLS IN PRIMED MICE
INTRODUCTION

The field of lymphocyte migration has recently been reviewed by Ford (1975). He has discussed the methods used for following migrating lymphocytes, the kinetics of T and B recirculation through lymph nodes and spleen, and the contribution of recirculation to primary and secondary responses. Relevant points will be considered briefly in this introduction.

Recirculation of T and B-cells; comparison of virgin and memory cells

Recirculation of lymphocytes from blood to lymph was first demonstrated by Gowans (1959). Previously, this had been indicated by the discovery that intravenous infusion of drained TDL (thoracic duct lymphocytes) was essential to maintain flow from a thoracic duct canula (Gowans 1957). In 1959, Gowans used \(^3\)H-thymidine to show that most TDL are not newly-formed cells, then labelled TDL with \(^{32}\)P to follow their passage after intravenous injection. Transit from blood to lymph occurs in the lymph nodes, lymphocytes entering the nodes via the endothelium of postcapillary venules (Gowans & Knight 1964) and leaving mainly via the efferent lymphatics (Hall & Morris 1962; 1963; 1964; 1965). Circulation also occurs through the spleen but lymphocytes probably enter and leave via vascular routes. Evidence is gradually accumulating that some cells, particularly primed cells, enter tissues not usually associated with lymphocyte recirculation. For example, memory cells may be detected in bone marrow and thymus, although the significance of these observations is uncertain (Benner et al 1974; Jahn & Karlin 1971; Micklem pers. comm.).

90-95% of TDL are small recirculating lymphocytes. The remaining 5-10% are blast cells which do not recirculate but home
to the gut (Gowans & McGregor 1965). 80-85% of TDL in mice (Sprent 1973) and 65-80% in rats (Parish & Hayward 1974) are T-cells, and the kinetics of their recirculation from blood to lymph has been studied in splenectomised rats (Ford & Simmonds 1972) using an $^{3}$H-uridine label which preferentially labels T-cells (Howard et al 1972). The majority were collected 14-18 hours after intravenous infusion. T-cell transit time across the lymph nodes is between 12-18 hours whilst that across the spleen is 2-6 hours (Ford 1969; Bradfield & Born 1973).

The properties of B-cells in the thoracic duct lymph have been determined by studying TDL from 'B' rats (Howard 1972), 'B' mice and athymic nu/nu mice (Sprent 1973). The thoracic duct lymph of 'B' rats contains only 20% as many lymphocytes as normal thoracic duct lymph, and these 20% are long-lived. However, there is evidence from functional studies of Strober (review: 1975) that some thoracic duct B-lymphocytes are short-lived and non-recirculatory. There is also some indication that recirculating B-cells traverse the spleen more slowly than do normal TDL (Sprent 1973). This is supported by the late peak in lymphocyte output during thoracic duct drainage of 'B' rats (Howard 1972). There is a steady flow of lymphocytes 18-48 hours after intravenous infusion, thus B-cells are probably responsible for the second low peak often seen when draining normal TDL. In mice the proportion of B-cells among the TDL after 12 hours drainage is 15%, but after 4 days drainage is 50% (Sprent 1973). Moreover at 3 days there is a depletion of splenic T-dependent areas but no depletion of B areas.

Recirculatory properties of lymphocytes may alter during their maturation. For example, antigen may be required to drive the
immature, short-lived and non-recirculatory T1 cell to a mature, long-lived recirculatory T2 cell (Raff & Cantor 1971; Araneo et al 1976). This implies that the T1 cell is a virgin T-cell while the T2 cell is a memory cell - a proposition which is not widely accepted. It has also been shown that at least some virgin T-cells recirculate (Ford 1975; Pearson et al 1976).

By definition, antigen is required to drive B-virgin cells to B-memory cells. There is some evidence that most virgin cells are short-lived and non-recirculatory, whilst memory B-cells are long-lived and recirculatory (Strober 1972). This was shown by passaging virgin or DNP-BSA primed rat spleen cells through an intermediate, X-irradiated recipient. Graded numbers of TDL collected from these intermediate host were injected into a second irradiated host together with a constant number of DT (dyptheria toxoid) primed helper cells, then these hosts were challenged with DNP-DT. A fixed number of passaged virgin cells gave a very much lower response than an equal number of unpasaged spleen cells. In contrast, passaged primed cells gave a better response than unpasaged controls (Strober & Dilley 1973; Strober 1975). Similar results were also obtained for virgin and memory cells to horse spleen feritin but virgin cells to SRBC were found to recirculate, possibly due to inadvertent environmental priming. Other instances of this were considered in Chapter 1. However it is not yet proven that an antigen-driven step is required to drive non-recirculating cells to recirculate. Evidence from TDL drainage of thymectomised foetal lambs indicates that some B-cells recirculate in the absence of any possible environmental priming (Pearson et al 1976).

Sessile memory cells
Although there is general agreement that memory T and B small lymphocytes recirculate, the existence of a sessile memory cell pool remains controversial. Chronic thoracic duct drainage of rats primed to SRBC, BSA or DT does not deplete their ability to give a secondary response upon challenge (McGregor & Gowans 1963; Phillips et al 1972), though it does reduce the secondary response to DNP (Feldbush & Gowans 1971). Passage of thoracic duct lymph containing primed cells through an intermediate host indicated that some of these cells settled out in the tissues since these hosts, in turn, could not be depleted of memory responsiveness by chronic TDL drainage (Ellis & Gowans 1973). However chronic drainage for 5 days cannot totally deplete the rat of recirculating lymphocytes, and even if only 5% remain, this should be sufficient to give a good secondary response upon challenge (Strober & Dilley 1973a). There is thus no need to postulate the existence of sessile memory cells.

There is considerable evidence for a sessile component of the memory cell pool being retained at the site of antigen priming, probably in response to persistent antigen. Experiments demonstrating this have involved priming an animal in one footpad and at intervals thereafter either

a) challenging the same animal in the draining node, contralateral node, or both, to compare the levels of response (Smith et al 1970; Stavitsky & Folds 1972);

or b) *in vitro* culture (Jacobson & Thorbecke 1969; Stavitsky & Folds 1972) or *in vivo* adoptive transfer (Ponzio & Spears 1973) of draining and contralateral nodes for challenge and comparison of responses.

The main difference between these two techniques is that the
latter measures the memory capacity of the lymph nodes at the time of removal whilst the former assays the memory content of the nodes plus any contribution from recirculating cells recruited by the challenge injection. However, similar results have been obtained using both systems. Rabbits primed to DT, BGG or KLH in one footpad developed a secondary response in the draining nodes within one week although memory levels continued to rise for several weeks (Jacobson & Thorbecke 1969; Stavisky & Folds 1972). No memory was detected in the contralateral nodes until at least 2-3 weeks after priming and differences between the memory capacity of draining and contralateral nodes were still apparent at 3-4 months. Similar studies in mice showed a secondary response to TT (tetanus toxoid) in the draining nodes within 10 days of priming but none was detected in the contralateral nodes until day 17 (Ponzio & Spears 1973). At day 17, the response of the contralateral nodes was 40 times lower than that given by the draining nodes, but by day 30 these responses had become equalised. Differences between secondary responses of nodes draining and contralateral to an SRBC priming injection have still been clear at 4 weeks after priming (Smith et al 1970). That this represents a pool of non-recirculatory memory cells is strongly suggested by the observation that memory to SRBC in draining nodes is resistant to pre-emption for at least 7-10 weeks after priming whilst that in non-draining nodes is not resistant (Inchley et al 1975).

The presence of active persistent antigen in lymphoid tissues has been implied not only by the memory experiments discussed above but also by the immediate rise of a late primary response if regulating antibody is removed from the serum (Graf & Uhr 1969). Early workers followed the fate of radioactive label in experimental animals.
after intravenous injection of $^{35}$S-KLH, BGG or BSA (review: Campbell & Garvey 1963). Label was detected in the liver and spleen for up to 3 years and at that time, the authors estimated that there were 2000 molecules of $^{35}$S-KLH associated with each liver cell. They also demonstrated a rough correlation between retained antigen and circulating antibody for several weeks after immunisation indicating that the antigen was retained in an active form for at least that long. Radioactivity has been detected in the lymph nodes for at least 2 months after immunising rats with particulate flagella and antigen which localised in lymphoid follicles retained its immunogenicity (Ada & Williams 1966). Antigen is usually found on the surface of follicular dendritic cells (Nossal & Ada 1971) and its localisation there is dependent upon antibody, particularly upon the Fc portion (Ada & Williams 1966; Herd & Ada 1969a and b).

Recent experiments have more clearly demonstrated that antigen retained in the lymph nodes after subcutaneous immunisation remains active for many months. When BSA primed (Tew et al 1973) or KLH primed (Stavitsky et al 1974) draining lymph node cells are placed in culture, even up to a year after priming, spontaneous antibody synthesis often occurs in the absence of any exogenously added antigen. This has been interpreted as being due to an antigen-specific stimulation of lymphocytes released from feedback control (Tew et al 1976). Greene et al (1975) showed an additive effect between "persistent" antigen and exogenously added antigen, in the levels of response induced. They suggest that although the level of spontaneous antibody synthesis is a function of the amount of persisting antigen, the maximum level obtained upon challenge is a function of the T and B cells present. These cells may be retained
by persistent antigen.

Information on the persistence of RBC antigens is not well documented although their persistence is implied by functional studies on memory cell generation and retention in draining nodes (Cunningham & Sercarz 1971; Smith et al 1974). Such studies as have been made involved a measure of the ability of persistent SRBC to initiate a primary immune response either after transfer of spleen extracts to irradiated recipients, or after irradiation and repopulation of the immunised animal (Franzl 1962; Ford 1968; Britton et al 1968). The results indicated that SRBC persists for only 3-14 days in an immunogenic form.

Long-term retention of memory cells is usually assumed to primarily affect B-cells since they possess both Ig receptors (Raff 1970) and Fc receptors (review: Dickler 1976) and can be absorbed out on fibroblast monolayers containing antigen (Ponzio et al 1975). There is no such evidence for helper T-cells, although they do have antigen receptors, but the studies of Miller (1973) showed that thymectomy reduced the non-specific component of responses given by long-lived sessile B-cells, and implied the existence of stationary T-memory cells.

It is known that antigen can select and retain specific cells for a short time after injection (Emeson & Thursh 1971; Thursh & Emeson 1972; Sprent et al 1971; Rowley et al 1972; Ford 1972; Ponzio et al 1975) in addition to the non-specific trapping effect (Hall & Morris 1965; Zatz & Lance 1971; Thursh & Emeson 1972). This is true for both T and B cells and may be related to the period of blast transformation (Ponzio et al 1975). TDL from rats primed intravenously to SRBC or TT (Rowley et al 1972) or mice primed
intravenously to SRBC (Sprent et al 1971) were temporarily depleted of the capacity to transfer a specific response to adoptive recipients, the refractory period lasting from 24-48 hours. Enrichment of the draining organ was more difficult to show and was best demonstrated by an in vitro challenge (Sprent & Lefkovits 1976) or by experiments such as the addition of TT primed cells to the perfusate of an isolated spleen primed to TT 3 hours previously, when the cells became detained in the spleen over the following 10 hours (Ford 1972). Emeson & Thursh (1971 and 1972) have studied the localisation of cells primed to heterologous erythrocytes and histocompatibility antigens. They labelled CRBC primed cells with $^3$H-thymidine and SRBC primed cells with $^{14}$C-thymidine (and vice versa), and studied the localisation of the two labels in a recipient primed with CRBC in one footpad and SRBC in the opposite footpad. There was always a predominance of the respective label in specifically primed nodes. Ponzio et al (1975) have shown a similar specific localisation of DNP-primed cells in response to follicular localised antigen-antibody complexes.

The mechanism of cell recruitment remains a mystery. It is widely believed that antigen-specific selection takes place within the node, and is rendered more effective by an increase in lymphocyte flow which causes the phenomenon of trapping (Cahill et al 1976; Hay & Hobbs 1977). In addition, it has been suggested that screening may occur at the post capillary venule (PCV), causing all specific cells to enter the node (McConnel et al 1974). Sheep primed to PPD (purified protein derivative of tuberculin) were depleted of responsiveness to that antigen by cannulation of the efferent lymphatic of a popliteal node accompanied by repeated local injections of PPD. Reactivity to other antigens was not depleted.
Aim of study

It is clear that antigen which persists in a draining lymph node, in some way affects memory cells in that node such that the memory capacity remains above that in the non-draining nodes for several weeks. Two mechanisms can be envisaged:

a) memory cells are totally sessile in the presence of antigen and, as antigen decreases in quantity with time after priming, these cells are gradually released into the circulation;

b) all memory cells have the capacity to recirculate but their passage through draining lymph nodes is retarded by contact with antigen.

The following experiments were designed in an attempt to distinguish between these two possibilities. It was thought that a gradual release of "sessile" memory cells in response to decreasing amounts of antigen might be reflected by increasing clonal heterogeneity in non-draining organs with time after priming. It seemed likely that low affinity memory cells would first be released and the clonal response of non-draining organs might be relatively restricted. With time after priming, a heterogeneous response may be expected in these organs and a restricted response of high affinity in draining nodes. Though it is possible that high affinity memory cells would be retarded in their passage more easily than low affinity cells, this is unlikely to produce clear clonal differences in non-draining organs. Thus, mice were primed in the two front footpads with SRBC, and at intervals after priming, draining and/or non-draining lymph node cells were adoptively transferred to irradiated recipients for challenge. Serum from
these recipients was focused and splenic plaque forming cells assayed to determine the memory capacity of draining and non-draining nodes.
MATERIALS AND METHODS

1. Animals, antigens, and PFC assays were as described in Chapter 2.

PFC assays in single nodes

In some experiments on recirculation of T and B cells, PFCs in a
single node were counted. Control mice were immunised bilaterally
and paired brachial nodes were assayed. In order to permit direct
comparison, individual PFC counts for all paired results in these
experiments were divided by 2 before calculation of geometric means.

2. Adoptive transfers, haemagglutination (HA) and IEF assays were
as described in Chapter 3.

3. Immunisation with RBC and DNP conjugates
   a) SRBC and CRBC

   Varying doses of SRBC or CRBC were usually injected subcutaneously
   (sc) into one or both front footpads. Hind footpads were used in
   some experiments for assay of the popliteal nodes.

   Where B. pertussis was incorporated with the priming RBC dose,
   5 x 10^8 organisms were divided between two footpads.

   Adoptive recipients were challenged with 10^8 SRBC iv.

   b) DNP_{0.2} CRBC

   5 x 10^7 DNP_{0.2} CRBC were injected sc into the two front or
two hind footpads with/without 5 x 10^8 B. pertussis. Adoptive
recipients were challenged with 10^8 DNP_{0.2} CRBC iv.

   c) DNP_{45}

   Either 50μg DNP-BGG were injected sc into the two front (or
rear) footpads with 5 x 10^8 B. pertussis or 25μg DNP-BGG were injected
sc into the left front (or rear) footpad with 2.5 x 10^8 B. pertussis.
Adoptive recipients were challenged with 50μg DNP-BGG iv.
RESULTS

Appearance of recirculating memory cells

Smith et al. (1970) primed mice in the left hind footpad with SRBC. Four weeks later these mice were challenged in both footpads and it was clear that the secondary response of the left node was higher than that of the right. There was some indication that the degree of difference was priming dose dependent.

The following experiments were designed to compare the responses of draining and non-draining nodes at different intervals after priming and adoptive transfer. Donor mice were injected with varying doses of SRBC sc in the 2 front footpads and at intervals after priming $10^7$ pooled draining lymph node cells (brachial and axillary) or non-draining lymph node cells (inguinal and popliteal) were transferred to irradiated (900r) recipients. Recipients were challenged with $10^8$ SRBC iv at the time of transfer. The kinetics of the adoptive primary and secondary response were first determined (Fig. 6.1) and as a result, a day 6 PFC assay was usually chosen in order to minimise the $1^\circ$ IgG PFC levels whilst allowing detection of peak $2^\circ$ IgG. It should be noted that the $2^\circ$ IgM PFC were falling at this time (particularly those primed only 7 days before transfer) whilst $1^\circ$ IgM were at their peak.

Fig. 6.2 shows the results of four experiments in which donor mice were primed with varying doses of SRBC 2 to 21 days before transfer and challenge. The capacity of draining lymph nodes to give a $2^\circ$ IgG PFC response was high by day 7 after all priming doses and remained relatively constant at this level for the next two weeks. Some memory had developed by day 4 after priming with $5 \times 10^5$ SRBC sc (Fig. 6.2a) but it continued to increase. In contrast, no IgG was detected in the
Fig. 6.1  Kinetics of the adoptive $1^0$ and $2^0$ response to SRBC.

PFC response in groups of 5 irradiated (900 r) recipients of $10^7$
pooled brachial and axillary lymph node cells and $10^8$ SRBCiv.
Donors were primed with $5 \times 10^6$ SRBCsc in the two front footpads
21 days ■■■■, or 7 days •••• before transfer, or
were unprimed ▲▲▲▲.
Fig. 6.2 Memory capacity in draining vs non-draining nodes: effect of priming antigen dose on recirculation of memory cells.

Dry 6 IgG PFC response in groups of 5 irradiated (900r) recipients of $10^7$ pooled brachial and axillary (O—O) or inguinal and popliteal (●——●) lymph node cells and $10^8$ SRBCiv. Donors were primed with (a) $5 \times 10^5$, (b) $10^6$, (c) $5 \times 10^6$ or (d) $10^7$ SRBCsc in the two front footpads 2-21 days before transfer.

* 4 mice died before assay.
Fig. 6.3 Memory capacity in draining vs non-draining nodes: effect of priming antigen dose on recirculation of memory cells.

Day 6 IgM PFC response in groups of 5 irradiated (900R) recipients of 10^7 pooled brachial and axillary (O-O), or inguinal and popliteal (●⋯⋯⋯●) lymph nodes cells and 10^8 SRBCiv. Donors were primed with (a)5x10^5, (b)10^6, (c)5x10^6 or (d)10^7 SRBCsc in the two front footpads 2-21 days before transfer.

* 2 mice died before assay.
non-draining nodes 7 days after priming, although some could usually be shown by day 10 or 14. By day 14 after priming with $5 \times 10^5$ SRBC sc draining and contralateral nodes gave equal responses. Following $10^6$, $5 \times 10^6$ and $10^7$ SRBC sc non-draining nodes gave secondary responses which were significantly lower than those of the draining nodes at day 14. However by day 21 after $10^6$ and $5 \times 10^6$ SRBC sc, draining and non-draining nodes gave equal responses. Differences between the two were, however, still significant after priming with $10^7$ SRBC sc. It therefore seems that IgG memory cell recirculation is dependent on the priming antigen dose and may be regulated by the amount of antigen persisting in each instance.

Secondary IgM responses are difficult to interpret (Fig. 6.3). Following the lower priming doses of $5 \times 10^5$ and $10^6$ SRBC sc no significant differences were seen between draining and non-draining nodes at day 7, 14 or 21 although differences were apparent four days after priming with $5 \times 10^5$ SRBC sc. After the higher doses of $5 \times 10^6$ and $10^7$, memory in the non-draining nodes was poor at day 7 and significantly lower than in the draining nodes. It rose by day 10 after $5 \times 10^6$ SRBC sc (Fig. 6.3c) and reached levels above that of the draining node before equilibrating with it. After a priming dose of $10^7$ SRBC sc, day 14 and 21 responses were almost equal to the draining node but remained slightly lower.

Persistence of memory

The ability of draining and non-draining nodes to give adoptive secondary PFC responses was followed for an additional 5-7 weeks after priming. Draining node memory remained constant at the day 7 levels over this period of time. Non-draining nodes, once equal to draining, continued to give responses indistinguishable from them.
Fig. 6.4 Memory capacity in draining vs non-draining nodes.

Day 6 IgG PFC responses in groups of 5 irradiated (900r) recipients of $10^7$ primed lymph node cells and $10^8$ SRBCiv. Donors were primed in the front footpads with $5 \times 10^6$ SRBCsc.

- $\bullet\bullet\bullet\bullet\bullet$, brachial and axillary cells transferred;
- $\bigcirc\bigcirc\bigcirc\bigcirc\bigcirc$, inguinal and popliteal cells transferred.

$\Delta^0$ response ± 1 se is also shown.
Fig. 6.5  Memory capacity in draining vs non-draining nodes.

Day 6 IgM PFC responses of groups of 5 irradiated (900r) recipients of $10^7$ primed lymph node cells and $10^8$ SRBCs. Donors were primed in the front footpads with $5 \times 10^6$ SRBCs.

- O- O, brachial and axillary cells transferred;
- ········, inguinal and popliteal cells transferred.

$1^0$ response ± 1 se is also shown.
(Fig. 6.4). IgG 2° responses followed a similar pattern to the IgG at 5-7 weeks in these experiments (Fig. 6.5).

Clonal memory

It has already been shown that a heterogeneous clonal spectrum is obtained from draining lymph node cells primed 7 days prior to transfer (Chapter 3). It was hoped that IEF analyses of these experiments on recirculation might reveal increasing clonal heterogeneity in the non-draining nodes with time after priming. Although PFC numbers in draining and non-draining nodes equalised early it seemed possible that non-draining nodes would contain only a few well-expanded clones. Intense spectra were seen in recipients of day 4 primed cells from nodes draining an injection of $5 \times 10^5$ SRBC sc but diversity was greater by day 14 (Fig. 6.6). In contrast, the day 4 non-draining nodes had very few bands, one clone only being detected in 2 out of 5 recipient mice. However by day 14 after priming the clonal spectra were as heterogeneous as in the draining nodes.

The results of two experiments in which donor mice were primed with $5 \times 10^6$ SRBC sc are shown in Figs. 6.7 and 6.8. Memory was developing by four days after priming and by day 7 the clonal response was heterogeneous. However, the responses of cells from non-draining nodes at both days 4 and 7 were still clonally restricted (Fig. 6.7). This was confirmed for day 7 responses (Fig. 6.8) and extended to include days 14 and 21 after priming. It is clear that at day 14 clonal memory was expanding in the non-draining nodes but was still more restricted than the draining nodes, whilst by day 21 the two responses were equally heterogeneous. The much stronger 2° response in the draining node given by 7 day primed cells compared with those
Fig. 6.6  Memory capacity in draining vs non-draining nodes: IEF assay.

IEF spectra of day 6 sera from irradiated(900r) recipients of (a) $10^7$ draining lymph node cells, or (b) $10^7$ non-draining lymph node cells, and $10^8$ SRBCiv. Donors were primed in the front footpads with $5 \times 10^5$ SRBC sc 2(A), 4(B) or 14(C) days before transfer.
IEF analyses of day 6 sera from irradiated (900r) recipients of $10^7$ draining(A) or non-draining(B) lymph node cells and $10^8$ SRBCiv.

Donors were primed in the front footpads with $5 \times 10^6$ SRBCse 4 days(a) or 7 days(b) before transfer.
Fig. 6.3 Memory capacity in draining vs non-draining nodes; IEF

IEF analyses of day 6 sera from irradiated (900r) recipients of 10^7 draining(A) or non-draining(B) lymph node cells and 10^8 SRBCiv. Donors were primed in the foot pads with 5x10^6 SRBC on 7(a), 14(b) or 21(c) days before transfer.
### TABLE I

Comparison of the adoptive 2° responses given by draining and non-draining lymph node cells at varying intervals after priming

<table>
<thead>
<tr>
<th>Interval between priming and transfer</th>
<th>Lymph nodes transferred</th>
<th>Haemagglutination titres (mean log$_{10}$ titre±s.e.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>21 days *</td>
<td>Draining</td>
<td>7.6 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>Non-draining</td>
<td>4.75 ± 0.75</td>
</tr>
<tr>
<td>14 days *</td>
<td>Draining</td>
<td>7.00 ± 0.30</td>
</tr>
<tr>
<td></td>
<td>Non-draining</td>
<td>5.80 ± 0.20</td>
</tr>
<tr>
<td>7 days **</td>
<td>Draining</td>
<td>7.60 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>Non-draining</td>
<td>3.80 ± 0.25</td>
</tr>
<tr>
<td>-</td>
<td>Pooled cells</td>
<td>3.25 ± 0.48</td>
</tr>
</tbody>
</table>

Groups of 5 mice were immunised with $5 \times 10^6$ SRBC sc in the 2 front footpads 7, 14, or 21 days before adoptive transfer of $10^7$ draining (brachial and axillary) or non-draining (inguinal and popliteal) lymph node cells to irradiated (900r) recipients for challenge with $10^8$ SRBC iv. Recipients were bled 7 days after challenge. Total and ZMER serum antibody titres were significantly higher in draining than in non-draining nodes at all times.

* $p < 0.05$  
** $p < 0.001$
Fig. 6.9  Memory capacity in draining vs non-draining nodes: IEF assay.

IEF analyses of day 6 sera from irradiated (900r) recipients of 10^7 draining(A) or non-draining(B) lymph node cells and 10^8 SRBCiv.

Donors were primed in the front footpads with 5x10^6 SRBCsc 1(a), 5(b) or 7(c) weeks before transfer.
primed 14 or 21 days before transfer should be noted. The reason for this fall in intensity when IgG PFC numbers (Fig. 6.2b) and haemagglutination (HA) titres (Table 1) remained constant are not clear. Discrepancies between PFC, IEF and HA data have already been discussed in Chapter 3 and will not be considered further here. After transfer of day 7 primed cells (Fig. 6.8a), a number of clones could be traced in all five recipients indicating that there were several memory precursors for each clone. This was less obvious in recipients of day 14 and 21 primed cells although it can be seen for some clones, for example mice 2 and 4 in Fig. 6.8c. It seems possible that recirculation of memory cells within the donor might limit the number of clones transferred and thus account for the loss of uniformity in recipients. However, remaining clones should have been well-expanded to explain the consistency of PFC levels and antibody titres and thus would have been expected to give intense IEF bands.

IEF analysis of sera from 1 week, 5 week or 7 week primed cells indicated that differences between the clonal spectra of recently primed cells and those primed for longer periods of time, were not always as apparent as in Fig. 6.8 (Fig. 6.9). Responses of non-draining nodes remained equal to those of draining nodes at 5 and 7 weeks, correlating well with the PFC data (Fig. 6.4).

Recirculation of memory cells after immunisation with other antigens

Memory generation to DNP and CRBC has been followed by priming with DNP$^{0.2}$ CRBC (Chapter 3). It seemed of interest to study the recirculation of memory cells to these two antigens and to compare it with that to SRBC. Donor mice were primed with $5 \times 10^7$ DNP$^{0.2}$ CRBC sc and the draining and contralateral nodes removed for adoptive transfer 4, 7, 14 or 21 days later. Recipient mice were challenged
with $10^8 \text{DNP}_{0.2}$ CRBC iv and their spleens were plaqued against both CRBC and DNP$_{0.04}$ SRBC on day 7 after transfer. PFC results are shown in Figs. 6.10 and 6.11. IgG memory to CRBC was found to be developed by day 7 in the draining nodes, rose during the next week then remained constant. It was first detected in the non-draining nodes 14 days after priming although it was significantly lower than in the draining nodes at that time. By day 21 it had risen to equal levels. IgM memory to CRBC was good in the draining nodes at day 7 and remained constant for the next two weeks. That in the non-draining nodes first appeared between days 7-14 and was equal to the draining node response at day 14.

As has already been shown in Chapter 3, memory to DNP is generated a little more slowly than to RBC antigens, first being clearly shown 21 days after priming (Fig. 6.11). Recirculation of these memory cells was unusual in that good memory, equal to the day 21 draining node response, was detected in non-draining nodes by day 14. This response remained constant for the following week. IgM memory to DNP was well-developed in the draining nodes by day 7, rose to day 14 then began to fall. That in the non-draining nodes was high by day 7, although almost 10 fold lower than in the draining nodes, and only rose slightly to day 21. At day 21, since the draining node IgM response was falling and the non-draining node response rising, differences between the two were diminishing although still statistically significant ($p < 0.001$).

It should be noted that IgG memory cells to both DNP and CRBC were established in the non-draining nodes at a time when memory in the draining nodes was still increasing.

Recirculation of T and B memory cells
Fig. 6.10 Memory capacity in draining vs non-draining nodes: recirculation of anti-CRBC memory cells.

Day 7 anti-CRBC PFC responses of groups of 5 irradiated (900r) recipients of 10^7 lymph node cells and 10^8 DNP-CRBCiv. Donors were primed with 5x10^7 DNP_{CRBC} + 5x10^8 B.pertussis in the front footpads at various intervals before transfer of draining O—O or non-draining ••••• lymph node cells.

1^0 response ± 1 se is also shown.
Days between priming and transfer

a. IgG

b. IgM

mean log₁₀ PFC ± se
Fig. 6.11 Memory capacity in draining vs non-draining nodes: recirculation of anti-DNP memory cells.

Day 7 anti-DNP PFC responses of groups of 5 irradiated (900R) recipients of $10^7$ lymph node cells and $10^8$ DNP$_{O_2}$CRBCiv. Donors were primed with $5\times10^7$ DNP$_{O_2}$CRBCsc + $5\times10^8$ *B. pertussis*, in the two front footpads, at various intervals before transfer of draining $\circ\circ$ or non-draining $\bullet\bullet\bullet\bullet$ lymph node cells.

$1^0$ response $\pm$ 1 se is also shown.
Days between priming and transfer

mean log_{10} PFC±se

a. IgG

b. IgM
Three weeks after priming to SRBC, CRBC or DNP a good secondary response can be seen on adoptive transfer of lymphocytes from nodes draining the antigen injection. Memory cells are also present in the non-draining nodes by this time and, upon adoptive transfer, these nodes give a secondary response equal, or almost equal, to that of draining lymph nodes. The ability to give an heterogeneous clonal response is a feature of both draining and non-draining nodes.

Discrepancies between these results and those of others showing differences between draining and non-draining nodes for many weeks after immunisation (Jacobson & Thorbecke 1969; Smith et al 1970; Stavitsky & Folds 1972) may reflect differences in the animals or assays used. In vitro cultures have been used to assay 2° responses in rabbit studies and should be analogous to these experiments (Jacobson & Thorbecke 1969; Stavitsky & Folds 1972). An in vivo adoptive transfer of tetanus toxoid (TT) primed cells from draining and non-draining lymph nodes of mice showed equality between the two by 30 days after priming. This indicates that the animal used may be important but Smith et al (1970) showed differences between the responses of draining and contralateral nodes of mice 4 weeks after priming to SRBC. It was decided to repeat this type of experiment using a hapten-carrier system to distinguish between recirculation of T and B memory cells. A time interval of 5 weeks was chosen to allow for decay of the primary response (Chapter 2) before challenge. At this time, memory in the non-draining nodes was shown to be high and equal to that in the draining nodes when measured after adoptive transfer (Fig. 6.4).

The experimental scheme is illustrated in Fig. 6.12. Mice were primed with DNP-BGG in the left footpad and CRBC in the right footpad.
EXPERIMENTAL SCHEME FOR STUDY OF
RECIRCULATION OF MEMORY T AND B CELLS

25 μg DNP-BGG + 2.5 x 10⁸ B. pertussis were injected SC into the left hind (or fore) footpad, and 5 x 10⁵ CRBC + 2.5 x 10⁸ B. pertussis into the right. 5 weeks later each footpad received a challenge injection of 2.5 x 10⁷ DNP-CRBC. Popliteal nodes were removed for PFC-assay 3 or 5 days after challenge.
This procedure ensures an adequate supply of DNP-primed B-cells in the left popliteal node and CRBC helper cells in the right node. Five weeks later both foopads were challenged with DNP CRBC. It should be possible to ascertain whether

a) CRBC primed cells have circulated to the left node to "help" in a 2° response to DNP

and

b) DNP-primed cells have circulated to the right node to give a secondary response to DNP.

In order to do this four control groups, each injected into both foopads with the same antigen(s), were essential. These are shown in Table 2. Typical day 3 and 5 PFC responses of these control groups are shown in Fig. 6.13. It is clear that the normal secondary response to DNP CRBC (Fig. 6.13a) was high by day 3 after challenge and remained at this level until day 5. In the absence of primed helper T-cells, as after priming with DNP-BGG alone (Fig. 6.13c), the 2° DNP response was slightly delayed and IgG PFC were significantly less than for the normal 2° response at day 3. The responses were equal by day 5. Provision of helper T-cells as well by additional priming with CRBC (Fig. 6.13b), brought the day 3 response up to the level of the normal 2° response. Carrier priming alone, on the other hand (Fig. 6.13d), induced no anti-DNP IgG PFC at all (and in fact suppressed the day 5 1° IgG response - Chapter 2). The IgM responses were very similar in all four groups.

These controls clearly showed that lymph nodes lacking primed T-cells gave a deficient 2° response at day 3 of assay. It follows that any primed T-cells recirculating to such nodes after priming elsewhere should enhance the day 3 response so that the response
**TABLE II**

Immunisation protocol for establishing the importance of primed B and T cells in a 2\(^\circ\) response to DNP-CRBC

<table>
<thead>
<tr>
<th>Priming injection (both footpads)</th>
<th>Anti-DNP memory cells</th>
<th>Anti-CRBC helper cells</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNP-CRBC</td>
<td>+</td>
<td>+</td>
<td>2(^\circ)</td>
</tr>
<tr>
<td>DNP-BGG + CRBC</td>
<td>+</td>
<td>+</td>
<td>2(^\circ)</td>
</tr>
<tr>
<td>DNP-BGG</td>
<td>+</td>
<td>-</td>
<td>?</td>
</tr>
<tr>
<td>CRBC</td>
<td>-</td>
<td>+</td>
<td>?</td>
</tr>
</tbody>
</table>

Groups of 5 female mice were primed in both front footpads, as shown, prior to challenge 5 weeks later with DNP-CRBC.
Fig. 6.13 The influence of primed T and B-cells on the response to DNP.

Mice were primed sc in each front footpad with (a) $2.5 \times 10^7$ DNP-CRBC, (b) $25 \mu g$ DNP-BGG + $5 \times 10^5$ CRBC, (c) $25 \mu g$ DNP-BGG or (d) $5 \times 10^5$ CRBC. 5 weeks later they were challenged with $5 \times 10^7$ DNP-CRBCsc for PFC assay of bronchial lymph nodes.

\[ \cdots \text{ IgM PFC response; } \cdots \cdots \text{ IgG PFC response. } \]

$1^0$ response ± se to DNP-CRBCsc is also shown.

N.B. All priming injections included $2.5 \times 10^8$ B. pertussis per footpad.
a. DNP-CRBC
B+T-cells

b. DNP-BGG+CRBC
B+T-cells

c. DNP-BGG
no T-cells

d. CRBC
no B-cells

mean log₁₀ PFC±se

Day of assay
becomes more typical of 6.13b than 6.13c. At the same time, lymph nodes lacking primed B-cells gave no IgG response on challenge with DNP-0.2 CRBC, and any primed B-cells recirculating to these nodes should produce a response more similar to 6.13b than 6.13d. The following experiments were performed to establish T and B cell circulation in this system, two with priming in the hind footpads and one with priming in the front footpads.

1) Do all memory T-cells freely recirculate?

The results of three experiments are shown in Figs. 6.14, 6.15 and 6.16, the first two involving priming in the hind footpads, the latter in the front footpads. One of the features of these experiments, particularly in groups primed with DNP-KGG + CRBC, has been the presence of one or two mice not producing IgG upon challenge. This has invariably been the cause of large standard errors where seen. Despite this, a consistent pattern emerged within these experiments. In Fig. 6.14, the experimental group (c) had a significantly higher day 3 IgG PFC response than the controls which were deficient in primed T-cells (d), indicating that some T-cells reached that node from the contralateral node primed with carrier alone. There are large standard errors in 6.14b on both days 3 and 5 so it is not possible to determine whether sufficient T-cells were present to give a maximum day 3 response. Fig. 6.15 shows an identical experiment in which once again IgG PFC levels in the experimental group (c) were significantly higher than in the control (d) and in this experiment, the day 3 IgG response shown in (c) was identical with that of mice primed with hapten and carrier on both sides (b). This infers that sufficient T-cells were present in the contralateral node by day 3 to help DNP-primed cells to give a peak 2° response.
Figs. 6.14 and 6.15

Do all memory T-cells freely recirculate?

Anti-DNP PFC responses, expressed per single popliteal lymph node, in groups of 5 mice challenged with $5 \times 10^7$ DNP-CRBCsc in both hind footpads 3 or 5 days before assay. Mice were primed sc in the hind footpads 5 weeks previously with:

(a) $2.5 \times 10^7$ DNP-CRBC in each footpad;
(b) 25µg DNP-BGG + $5 \times 10^5$ CRBC in each footpad;
(c) 25µg DNP-BGG in the left footpad and $5 \times 10^5$ CRBC in the right;
(d) 25µg DNP-BGG in each footpad.

All priming injections included $2.5 \times 10^8$ B.pertussis per footpad.

\[ \rightarrow \] , IgM PFC; \[ \bullet \bullet \] , IgG PFC.
Fig. 6.14

a. 1°

— 2° B+T-cells

b. DNP-BGG+CRBC

B+T-cells

c. DNP-BGG

? T-cells

d. DNP-BGG

no T-cells

Day of assay
Fig. 6.15

a. 1°

--- 2° B+T-cells

b. DNP-BGG + CRBC

B+T-cells

c. DNP-BGG

? T-cells

d. DNP-BGG

no T-cells

Day of assay
Fig. 6.16 Do all memory T-cells freely recirculate?

Anti-DNP PFC responses expressed per single brachial lymph node, in groups of 5 mice challenged with $5 \times 10^7$ DNP-CRBC sc in both front footpads 3 days before assay. Mice were primed sc in the front footpads 5 weeks previously with:

(a) $1 \times 10^6$ anti-DNP response to $2.5 \times 10^7$ DNP-CRBC sc;

(b) $2.5 \times 10^7$ DNP-CRBC in each footpad;

(c) $25 \mu g$ DNP-BGG + $5 \times 10^5$ CRBC in each footpad;

(d) $25 \mu g$ DNP-BGG in the left footpad and $5 \times 10^5$ CRBC in the right;

(e) $25 \mu g$ DNP-BGG in each footpad.

$2.5 \times 10^8$ B. pertussis per footpad were included with all priming injections.

\[ \text{IgM PFC; IgG PFC.} \]
Exactly the same results were obtained after priming in the front footpads (Fig. 6.16). The left node primed with DNP-BGG gave a response equal to controls primed with DNP-BGG and CRBC, and above controls primed with DNP-BGG alone. These experiments clearly indicate that CRBC helper cells recirculated from the right brachial nodes and were equally available to both draining and non-draining nodes.

2) Do all memory B-cells freely recirculate?

The results of three experiments are shown in Figs. 6.17, 6.18 and 6.19, the first two involving priming in the hind footpads and the latter in the front footpads. In all three experiments there was an IgG response at day 3 of assay in the experimental group (c). This group, primed with CRBC only in the right footpad, could not have given an IgG response unless DNP-specific memory cells, generated in the left node, had recirculated to the right. Responses of popliteal nodes were low, and variability between animals was large, but the brachial nodes gave good responses with small standard errors. In each experiment the day 3 response of the experimental group (c) was lower than that of the control group (b) although this was only statistically significant in the experiments shown in Figs. 6.18 and 6.19. It would thus seem that although B-memory cells were present in the contralateral nodes and could give an IgG response at day 3, there were not sufficient present to give a response equal to that of the draining nodes of control groups. By day 5 there was no difference between this group and the controls (c and b) though this was only clear in Fig. 6.19 where standard errors were low.
Figs. 6.17, 6.18 and 6.19

Do all memory B-cells freely recirculate?

Anti-DNP PFC responses expressed per single lymph node in groups of 5 mice challenged with $5 \times 10^7$ DNP-CRBC in both footpads 3 or 5 days before assay. Mice were primed sc in the hind (Figs. 6.17 and 6.18) or front (Fig. 6.19) footpads 5 weeks previously with:

(a) $2.5 \times 10^7$ DNP-CRBC in each footpad;
(b) $25 \mu g$ DNP-BGG + $5 \times 10^5$ CRBC in each footpad;
(c) $25 \mu g$ DNP-BGG in the left footpad and $5 \times 10^5$ CRBC in the right;
(d) $5 \times 10^5$ CRBC in each footpad.

$2.5 \times 10^8$ B. pertussis per footpad were included with all priming injections.

▼▼, IgM PFC;  ●●, IgG PFC.
Fig. 6.17

a. 1°

--- 2° B+T-cells

--- B+T-cells

b. DNP-BGG+CRBC

c. CRBC

? B-cells

d. CRBC

no B-cells

Day of assay
Fig. 6.18

a. \( \cdots 1^\circ \)  
\( \cdots 2^\circ \) B+T-cells

b. DNP-BGG+CRBC  
B+T-cells

c. CRBC  
? B-cells

d. CRBC  
no B-cells

mean log\(_{10}\) PFC+se

Day of assay
Fig. 6.19

a. **** 1°
   
   2° B+T-cells
   
   [Graph showing data with error bars]

b. DNP-BGG+CRBC
   
   B+T-cells
   
   [Graph showing data with error bars]

c. CRBC
   
   ? B-cells
   
   [Graph showing data with error bars]

d. CRBC
   
   no B-cells
   
   [Graph showing data with error bars]
DISCUSSION

The first series of experiments (Fig. 6.2 - 6.11) were designed to study the memory capacity of lymph nodes draining and contralateral to the site of an sc SRBC injection after different intervals of time. An in vivo adoptive transfer system was chosen to exclude any effects of feedback antibody on expression of the 2° response, and also to exclude any contribution of memory cells recruited from the recirculating pool in response to the challenge injection.

These experiments indicated that memory to SRBC developed in the draining nodes between days 4-7 after priming and remained constant at this level for the next 5-6 weeks (see Chapter 3). Memory in the non-draining nodes was first detected in cells transferred 10-14 days after priming. It continued to increase in these nodes to equal that of the draining nodes by 21 days after priming. There is some evidence that this recirculation was antigen priming dose dependent (Fig. 6.2). Non-draining nodes had a memory capacity equal to that of draining nodes within 14 days of priming with $5 \times 10^5$ SRBC while after priming with $10^7$ SRBC there were still differences at day 21. Support for this conclusion also came from IEF analyses of antibodies produced by cells after priming with different doses of SRBC (Figs. 6.6 and 6.8). This might be expected if memory cells are retained in the draining lymph node by persistent antigen. A similar dose dependence has been illustrated in other systems. Smith et al (1970) primed mice in the left footpad with $2 \times 10^6$, $2 \times 10^7$ or $2 \times 10^8$ SRBC sc. Four weeks later they were challenged with the same dose range in both footpads. There were differences in secondary responses between draining and contralateral nodes but after the lower priming dose ($2 \times 10^6$) differences were only 2 fold,
whilst after the higher priming doses (2 x 10^7 and 2 x 10^8) nearer 10 fold differences were seen. These were particularly marked after a low dose challenge. Inchley et al (1975) showed a dose dependence in studies on the resistance of "sessile" (draining node) memory cells to pre-emption. Following priming doses of 5 x 10^5 and 5 x 10^7 SRBC sc the day 22 2^o response in those nodes could not be pre-empted by SRBC ip. However the day 22 response to 5 x 10^3 SRBC sc could be pre-empted. It should be noted that a dose of 5 x 10^5 SRBC sc which cannot be pre-empted at 3 weeks has an excellent recirculating memory pool by this time (Fig. 6.2).

If release of memory cells is dependent upon the amount of antigen persisting in the draining lymph node, then it might be expected that low affinity clones would first be released, and that with time after priming the clonal response in non-draining nodes would become increasingly heterogeneous. There is some evidence for increasing clonal heterogeneity in the non-draining nodes between days 7, 14 and 21 after priming (Fig. 6.8). The clonal spectrum of the draining nodes did not alter in diversity but individual bands became reduced in intensity with time after priming. Since PFC and haemaglutination titres did not reflect this, it is possibly related to antibody affinity, perhaps indicating that more high affinity memory cells were present early after priming. This would not be expected if low affinity cells were first released into the circulation leaving only higher affinity cells in the draining node. The latter is pure speculation but might be tested in hapten-carrier studies by PFC inhibition.

It should be noted that as recirculating memory cells increased in number there was no corresponding decrease in the memory capacity of the draining lymph nodes. This must be explained either as an
artefact of the transfer system allowing plateau responses to be reached by a threshold number of cells, or by continued generation of memory cells. It is difficult to explain the constant response in the draining node if the latter is true. However recirculating cells specific for CRBC or DNP were detected in non-draining nodes at a time when the draining node response was still increasing (Fig. 6.10 and 6.11).

It could be argued that antigen carry-over from the draining nodes was instrumental in inducing memory generation in other lymphoid organs. Antigen could reach the contralateral nodes either in the form of antigen-antibody complexes, or carried on the surface of early released memory cells (Hall et al 1967; Greene et al 1975). If this were so, the memory capacity of non-draining nodes would not provide an accurate reflection of memory cell recirculation. There is not sufficient antigen carry-over to stimulate a primary response in contralateral nodes (Hall et al 1967, Jacobson & Thorbecke 1969; Smith et al 1970; Stavitsky & Folds 1972; Weissman et al 1973). However it is known that memory cells can be generated with lower doses of antigen than are required for stimulation of a primary response (Chapter 3). In studies of spontaneous antibody synthesis in vitro many weeks after priming to HSA, Greene et al (1975) have shown that draining nodes give a 100 fold higher response than unprimed controls, whilst non-draining nodes only give a 3 fold higher response. If this is, indeed, related to the amount of antigen persisting in the lymph node, it indicates that only low levels reach the contralateral nodes.

Thorbecke and Bell (1973) have approached this problem in a different way, by injecting passive antibody (anti-DT) into the contralateral node of a rabbit to prevent any priming in that node
by carried-over antigen. They showed that this treatment increases the difference in responsiveness between draining and contralateral nodes. However it was never absolute and at least 50% of the memory in contralateral nodes must have been due to recirculating memory cells. It is equally possible that passive antibody masks the antigen such that recruitment of recirculating memory cells becomes impaired (Rowley et al. 1972). Memory cells home specifically to the node draining an antigen injection (Emeson & Thursh 1971; Thursh & Emeson 1972) but on the other hand antigen-antibody complexes have also proved efficient at localising memory cells in lymphoid follicles (Ponzio et al. 1975) so it appears that the effect of passive antibody on residual antigen might be to improve localisation but to reduce priming.

Studies on the homing properties of T and B memory cells in the absence of antigen have been carried out by transferring primed cells to irradiated recipients. T-memory cells home preferentially to the lymph nodes whilst B-memory cells home primarily to the spleen (Romano et al. 1975; Cantor 1971). Therefore, in the present work it might be expected that non-draining nodes would be deficient in B-cells since they would tend to home to the spleen after leaving the site of priming. Since memory responses in the non-draining nodes were good, this imbalance is unlikely, although it is possible that memory T-cells combine with virgin B-cells to give the secondary response in the non-draining nodes.

Many workers have found that the lymphoid organs draining the site of a priming injection give higher $2^{°}$ responses than non-draining organs for many weeks after priming (Jacobson & Thorbecke 1969; Smith et al. 1970; Stavitsky & Folds 1972; Benner et al. 1974;
This has already been considered in the introduction to this chapter. There is no obvious reason why the results of transfer experiments presented in this chapter should differ from these other results. However, differences could relate to the technique since Ponzio and Spears (1973) found equality by day 30 between nodes draining and contralateral to an sc TT injection in mice, when assayed by adoptive transfer. For this reason, it was decided to follow the recirculation of memory cells in a system similar to that used by Smith et al (1970) to study the SRBC memory capacity in mice. A hapten-carrier system was chosen to allow distinction between T and B-cell recirculation. It is generally assumed that B-memory cells are retained by persistent antigen while T-cells are released to freely recirculate after blast transformation. There is, however, some evidence that memory T-cells also can be retained at the site of antigen injection (Miller 1973).

The result showed that sufficient memory T-cells were present in the contralateral node 5 weeks after priming to help primed B-cells give a day 3 IgG PFC response equal to mice primed with CRBC in that node (Figs. 6.14, 6.15 and 6.16). On the other hand, memory B-cells, although present in the contralateral node in sufficient numbers to give a day 3 IgG PFC response, did not give a response equal to that of the draining node. By day 5 these responses were rising and sometimes equal (Figs. 6.17, 6.18 and 6.19).

The possibility that antigen reached the contralateral node to prime T-cells cannot be totally eliminated and has already been discussed. Popliteal nodes were originally chosen for study as antigen is less likely to "leak" to the contralateral popliteal node than to the contralateral brachial. Results from popliteal and brachial studies were, in fact, similar. Smith et al (1970) found a
similar 24 hour delay in the contralateral node 2° response to SRBC. They showed that this delay could be attributed to the time required for recruitment of memory cells into the lymph node and could be eliminated by an injection of goose RBC 24 hours before challenge. It is surprising that there is not a similar lag in T-cell recruitment but this may be explained by the more rapid recirculation kinetics of T-cells (Ford & Simmonds 1972; Sprent 1973).

If the delay in the secondary response of contralateral nodes was related to a recruitment of lymphocytes, then the prompt response in the draining node must be explained by one of the following:

a) Some memory cells were still resident in the draining node at 5 weeks after priming and could give a prompt response with a day 3 peak.

b) All memory cells were freely recirculating by 5 weeks after priming but passage through the draining node was retarded by persistent antigen, so that at any one time the draining node would contain more memory cells than the contralateral node.

It is not possible to distinguish between these two possibilities from these experiments. It has been proposed that the enlargement of primed lymph nodes (review: Ford 1974) may be sufficient to allow more cells to pass through at any one time thus accounting for differences in response between primed and non-primed nodes. In these experiments both nodes were primed and thus enlarged so this may have accounted for the equal day 5 response not observed by others (Smith et al 1970).

The inevitable conclusion from these experiments is that T and B-memory cells freely recirculate and can give a good response in non-draining nodes 5 weeks after priming. At the same time there appears to be a "sessile" memory population primarily resident within
the draining nodes. These cells may be truly sessile or recirculating cells retarded in their passage. It is difficult to account for the differences between these results and those of adoptive transfer experiments since, by eliminating cell recruitment, differences between the nodes would be expected. It would seem that the adoptive response might be too insensitive to detect such differences. Unfortunately it would not be possible to study the draining and contralateral responses in vivo earlier than 5 weeks because of the ongoing primary response and high levels of circulating antibody.

One further point of interest arises from the adoptive transfer experiments, namely the reason for the absence of memory in the non-draining nodes at day 7 after priming. A similar delay has been shown in T-cell recirculation from an SRBC-primed spleen, primed T-cells predominating in the spleen at days 4-7 and in the lymph nodes by day 14 (Romano et al 1975). However, memory cells, both T and B, are released into the circulation from the spleen and can be detected in the thoracic duct lymph 3-5 days after intravenous priming. By 5-10 days enhanced responsiveness of the TDL is apparent (Rowley et al 1972; Sprent 1973). It is likely that the specific retention within the spleen and lymph nodes which lasts for 1-2 days, coincides with blast transformation of T and B-cells (Ponzio et al 1975). However, it is surprising that if memory cells can be detected among the TDL by adoptive transfer of day 5 cells, none can be detected in the contralateral nodes at this time.
CONCLUDING REMARKS
CONCLUDING REMARKS

In the last decade isoelectric focusing has proved a useful technique for establishing the clonal heterogeneity of antibody responses. It also gives insight into the size and nature of B-cell clones recruited into a response when used in conjunction with other techniques measuring the amount and affinity of serum antibody (haemagglutination, hemolysis and Farr assays) and the number of antibody forming cells (PFC assay). In this project the technique was used to investigate certain aspects of priming and memory which benefit from study at the level of individual clones due to the complexity of the overall response.

Phillips and Dresser (1973a and b) described a technique for detection of focused anti-SRBC antibodies by lysis of SRBC incorporated in a red cell overlay of the gel, and asserted that this was an extremely sensitive assay. This procedure has been used in these experiments but the IEF spectra so obtained were sometimes hard to correlate with PFC results and left some doubt as to the sensitivity of the assay with respect to low affinity antibody (see Chapter 3). Closer analysis of the developing sera used for the two assays, and more extensive correlation with haemagglutination and haemolysis assays, would clarify this work.

Despite these limitations, IEF analysis of the anti-SRBC response proved a useful approach to the study of the recruitment of B-cell clones into the memory cell pool (Chapter 3) and to the dissemination of memory cells throughout the body (Chapter 6). In recent years, other workers have also used isoelectric focusing to study the kinetics and requirements of memory generation (Askonas & Williamson 1972; Askonas et al 1972; Askonas & Roelants 1974;
These experiments involved adoptive transfer of dominant anti-DNP clones through several generations of irradiated recipients. Memory to DNP was fully established within a single clone within 7 days of priming and did not increase thereafter (McMichael & Williamson 1974). This stands in contrast to the results of others (reviewed in Chapter 3) which indicated that memory to many antigens continued to increase for several weeks or months after priming, but may have been a result of memory regeneration within a primed B-cell line as opposed to de novo generation from virgin cells. However, the continued rise in memory demonstrated in the intact animal may be explained by newly recruited memory clones since it has been shown that the clonal content of the memory cell pool undergoes changes even after the pool has reached a maximum size (Kreth & Williamson 1973).

These studies of Kreth and Williamson (1973) involved adoptive transfers of limiting numbers of cells such that each recipient expressed only 1-3 clones. This is an expensive and time-consuming assay but useful in that it allows a quantitative measure of clone size to be gained from the IEF technique. Limiting dilution analyses of cells in microcultures would prove extremely valuable, but as yet microculture supernatants have not been successfully focused despite the demonstration of IgG PFC and anti-SRBC lysis in agar. However it may prove possible if the sensitivity of the assay was further increased. One promising approach to the increase in sensitivity which is at present being tested in our laboratory, is the use of enzymes such as lactic dehydrogenase (LDH), as antigens. It has also proved possible to immunise animals with NIP-LDH (Melchers et al 1973), thus there is no obvious reason why
haptenated enzymes should not be used to increase the sensitivity of detection of electrofocused anti-hapten antibodies.

Haptens induce responses of considerably greater homogeneity than do complex antigens, such as proteins and RBC and are thus to be preferred as immunogens for clonal studies. Recently, homogeneity of anti-hapten responses has been further increased by the use of synthetic carrier molecules (Civin et al. 1976). The hapten, DNP, was chosen for the present work, chiefly because it has been extensively used in other clonal memory studies (Askonas & Williamson 1972; Askonas et al. 1972; Askonas & Roelants 1974; McMichael & Williamson 1974) and because the DNP-RBC overlay was successful for detection of electrofocused anti-DNP antibodies (Phillips & Dresser 1973b). However, two problems with DNP occurred during the project. Firstly, the natural splenic background PFC response to this hapten is usually fairly high, and work of Strober (review: 1975) has indicated that "virgin" anti-DNP B-cells may have had some contact with cross-reacting antigen. Secondly, detection of electrofocused antibodies by the DNP-SRBC overlay technique proved unsuccessful in this work due to the instability of the covalently conjugated DNP-SRBC in the presence of certain batches of guinea-pig complement. This may have been due to anti-DNP antibody in the complement but absorption with DNP-sepharose proved ineffective. Coupling with haptenated anti-Fab fragments (Phillips & Dresser 1973b) may yield more stable DNP-RBC thereby accounting for success in the IEF overlay. As an alternative, a radioautographic technique was established according to the method of Keck et al. (1973). The technique is similar to that described by Williamson (1971) and
initial results were good. It also has the advantage that antibody is fixed in the gel before addition of labelled antigen; thus it can be adapted for use with protein antigens since it reduces the hazard of diffusion of antibody during the extensive binding process.

In Chapter 2 certain aspects of hapten-carrier responses were studied and immunisation with DNP-RBC and DNP-protein conjugates was compared. Immunisation with haptenated RBC is a novel approach which has only been used in recent years and with limited success. One of the disadvantages is the lack of a method for estimation of the epitope density on the red cell surface. However, even with respect to hapten-protein conjugates where epitope densities are accurately determined, conceptions of "high" and "low" density differ markedly. It seems of more value to establish the IgM/IgG ratio of a response following different degrees of conjugation than to determine exact epitope concentrations. With the establishment of a sensitive anti-hapten assay for IEF, many of these experiments could be usefully extended. There was little difference in the size of the anti-DNP PFC response following immunisation with "low" and "high" density conjugates of DNP-CRBC. However differences may have been apparent in the clonal spectra, the lower density conjugate perhaps inducing a more heterogeneous spectrum due to increased anti-NAD antibody production. PFC inhibition studies should provide similar information. Likewise, IEF analysis of secondary response following challenge with either the same or a different conjugate might prove useful with respect to the in vivo determination of clonal heterogeneity.

In the context of studying the kinetics of priming (Chapter 3), the present studies need to be extended to follow variations in
clonal content of the memory pool with time after priming (Kreth & Williamson 1973). At present the only available approach to this study is that of in vivo limiting dilution analysis. It would therefore be essential to restrict the study to haptens (DNP) since the anti-SRBC response is highly heterogeneous, and identification of individual clones would prove extremely difficult. If there is a continual turnover of individual memory clones after the memory pool has reached a maximum size, as has been suggested by Kreth & Williamson (1973) for the anti-NIP response, then it needs to be determined whether this is related to the antigen used for priming (RBC or protein conjugate) or to its persistence in an immunogenic form. Affinity studies would again provide a useful complement to IEF analysis.

Recirculation studies, involving adoptive transfer, should also be extended using hapten-carrier conjugates, to compare T and B recirculation and to determine the effect of the nature and persistence of different antigens on the dissemination of memory. An in vitro assay may eliminate the artefacts arising from adoptive transfer.

It is essential to use an adjuvant to obtain a response to hapten-protein conjugates, hence the value of immunising with DNP-RBC conjugates to determine any effect the adjuvant may have on the nature of the response given. The work in Chapter 2 indicated that the adjuvant, B. pertussis, increased the magnitude of the response to DNP-CRBC but had no effect on the IgM/IgG ratio or on the kinetics of the response. Nevertheless, the adjuvant work in Chapter 5 should be extended to a study of the effects of C. parvum and B. pertussis on the clonal response to hapten-carrier conjugates.
This approach would indicate much more clearly whether the adjuvant effects are on B-cells, T-helper cells, T-suppressor cells or macrophages, particularly if synthetic polymers, rather than natural proteins or red blood cells, were used for hapten conjugation.
ACKNOWLEDGEMENTS

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APPENDIX

1. **BIOZZI PBS pH 7.2**

\[
\begin{align*}
\text{KH}_2 \text{PO}_4 & \quad 0.86\text{g/l} \\
\text{Na}_2 \text{HPO}_4 \cdot 12\text{H}_2\text{O} & \quad 5.69\text{g/l} \\
\text{NaCl} & \quad 5.67\text{g/l}
\end{align*}
\]

Equivalent to 1:2 ratio of phosphate buffer pH 7.2 and 0.85% NaCl.

2. **DUTTONS BALANCED SALINE (DBS)**

**STOCK 1**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10g dextrose</td>
<td>1.86g CaCl(_2\cdot 2\text{H}_2\text{O})</td>
</tr>
<tr>
<td>0.60g \text{KH}_2\text{PO}_4</td>
<td>4.00g KCl</td>
</tr>
<tr>
<td>4.78g \text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}</td>
<td>80.00g NaCl</td>
</tr>
<tr>
<td>20mls phenol red (0.02%)</td>
<td>2.00g MgCl(_2\cdot 6\text{H}_2\text{O})</td>
</tr>
</tbody>
</table>

**STOCK 2**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.00g MgSO(_4\cdot 7\text{H}_2\text{O})</td>
</tr>
</tbody>
</table>

Each solution made up to 1000mls with distilled water.

Working solution: 800mls distilled water + 100mls each stocks (1) and (2).

3. **TRIS buffer**

6g Tris (2-amino-2 (hydroxymethyl) propane-1, 3-diol
6g NaCl
5ml Ca-Mg stock*
900mls H\(_2\text{O}\)
IN HCl \(\rightarrow\) pH 8.0
Final volume 1 litre

*Ca-Mg stock = 4.3mg CaCl\(_2\cdot 2\text{H}_2\text{O}\) + 20.39g MgCl\(_2\cdot 6\text{H}_2\text{O}\)/litre.

4. **TRIS-glycyl-glycine (TRIS-gly-gly) pH 8.0**

TRIS buffer pH 8.0 + 10mg/ml glycyl-glycine
2N NaOH \(\rightarrow\) pH 8.0

5. **TRIS + glucose + BSA (TRIS + D + BSA) pH 7.4**

TRIS buffer
+ 0.5% dextrose
+ 1.0% BSA (Fract V)
IN NaOH \(\rightarrow\) pH 7.4
6. Preparation of gels for isoelectric focusing

47.4 ml distilled H₂O
3.0 ml Ampholines pH 5-8 (LKB Ltd.)
9.0 ml Acrylamide solution*

Degassed for approximately 3 minutes, then
0.3 ml 10% TEMED solution (NNN' tetramethylene diamine)
and 0.3 ml 10% Ammonium persulphate

were added, and the solution immediately poured into moulds.
Perspex moulds were made by the Zoology Department Workshop,
Edinburgh. Thickness = 0.5 mm.

*Acrylamide solution

33.3 g Acrylamide (BDH Chemicals Ltd. Poole)
0.9 g recrystallised bisacrylamide (BDH Chemicals Ltd. Poole)
Distilled water to a final volume of 100 ml.

Dissolve acrylamide and recrystallised bisacrylamide in 50 ml
distilled H₂O with aid of magnetic stirrer in fume cupboard
(30 mins.). Determine volume (N.B. acrylamide expands in
solution) and add distilled H₂O to a final volume of 100 ml.
Mix, then filter through Whatman No. 1 filter paper.

Store in dark. Keeps 1 month.

Recrystallisation of NN-methylenebisacrylamide

11 g NN-methylenebisacrylamide
1 litre Analar acetone

(a) Dissolve bisacrylamide in acetone, in a flask in a water bath
    heated from 0°C → 50°C in fume cupboard. Maintain at
    50°C for 60 mins.

(b) Filter while hot using warmed filter funnel and Whatman filter
    paper.

(c) Allow to cool overnight at 4°C, then at -20°C. Recover
    crystals by filtration (i.e. filter off fluid from crystals
    formed in cold, using cold funnel and paper)
(d) Wash crystals briefly in filter funnel and dry either by
   (i) spreading on a large sheet of chromatography paper
       (or filter paper) and covering with a second sheet
to keep dust free
   or (ii) in an oven at 40°C.

   Drying is complete when sheets no longer smell of acetone.
REFERENCES


191.


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Leyden. Ed. van Furth.
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