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Role of T cells in the development of memory B cells. Quantitative and qualitative analysis

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Summary. The purpose of this investigation was to address the current controversy regarding the T-cell requirement for the generation of B-memory cells. We have circumvented the possible objection to previous experiments regarding residual T cells in T-deprived animals by examining memory cell generation in relation to the numbers of T cells participating in the immune response.

Thymectomized and lethally-irradiated rats were reconstituted with foetal liver or a more mature stem cell source, neonatal liver. These animals were given graded doses of purified T cells one day before immunization with alum-precipitated DNP-BGG + *Bordetella pertussis*. Four weeks after priming, cell suspensions from experimental groups were adoptively transferred to carrier primed recipients and challenged with DNP-BGG in saline to assess memory cell development. The data demonstrate that in the absence of T cells only minimal memory development occurred. However, when T cells were present, the level of memory cell development increased with increasing numbers of T cells. By examining the relative affinity of the antibody produced in the primary and secondary responses, the increase in memory cell development in relation to increased numbers of T cells was shown to be due to the selective generation of high affinity memory B cells.

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INTRODUCTION

The injection of antigen elicits both a primary humoral immune response and the generation of memory cells. The differentiation of antigen-specific B cells into antibody-forming cells in the primary response is known to require the participation of T lymphocytes for many antigens. However, the requirement for T cells in the generation of memory cells is still controversial.

Previous investigators utilizing animals presumably depleted of T cells have provided evidence both for and against T-cell involvement in the generation of memory cells. Adult animals which were thymectomized, lethally irradiated, and bone marrow reconstituted were not able to develop a second response to poly-L-(Tyr-glu) (Mitchell, Grumet & McDevitt, 1972), BGG (Williams & Waksman, 1969) or TNP-*Brucella* (Romano & Thorbecke, 1975). Adult thymectomy alone also resulted in a decreased ability to develop a secondary response to bovine serum albumin (BSA) or ovalbumin (OVA; Simpson & Cantor, 1975). It may be argued that these experimental systems only show the inability to express a secondary response since it has been shown that T cells are needed for the differentiation of memory B cells to

plasma cells (Miller & Sprent, 1971). This criticism was overcome by employing the adoptive transfer of cells from putative immunized donors to carrier-primed recipients. In this adoptive transfer system it has been reported that memory cells were either not generated or appeared in only low numbers in congenitally-athymic mice (Schlegel, 1974; Dresser & Popham, 1979), and in anti-lymphocyte serum-treated mice (Braley-Mullen, 1977). In allotype suppressed animals, which have been shown to have allotype-specific T-suppressor cells and lack allotype specific T_H cells, a memory response was shown upon adoptive transfer to carrier-primed recipients, but this response seemed to be due to only low-affinity memory cells (Okamura, Metzler, Tsu, Herzenberg & Herzenberg, 1976). Therefore, one would conclude that memory cells, at least high-affinity memory cells, may not develop in the absence of T cells. In contrast, other investigators using congenitally-athymic mice showed memory responses when cells from these animals were challenged *in vitro* in the presence of T cells (Diamantstein & Blitstein-Willinger, 1974) or in the presence of supernatants from antigen-stimulated T cells (Schrader, 1975). Furthermore, it has been reported that adult thymectomized, lethally-irradiated and bone marrow-reconstituted animals develop a secondary response upon adoptive transfer (Shinohara & Tada, 1974) or when thymocytes were given at the time of antigen challenge (Roelants & Askonas, 1972). Since these experiments utilized bone marrow cells as a B-cell source, it is possible that low levels of T cells may have been present and thus explains the contradictory results reported.

A better approach to this question would be the use of T cell-deficient animals reconstituted with graded numbers of mature T cells. If T cells are needed for the generation of memory cells, it would be predicted that the memory response would increase with increasing numbers of T cells participating in the response. This premise circumvents the possible objection to previous experiments regarding residual T cells in T-deprived animals. This type of experimental design also allows for the evaluation of the role of T cells in the selection of high-affinity memory B cells. In primary responses both the affinity and heterogenicity of the antibody-response increase as a function of time (Werblin & Siskind, 1972; Werblin, Kim, Quagliata & Siskind, 1973; Kim & Siskind, 1974). In the absence of T cells this maturation apparently does not occur (Gershon & Paul, 1971; DeKruyff & Siskind, 1979). Previous studies have suggested that T cells are also required for high-affinity memory-cell development (Okamura *et al.*, 1976) but these results have only been reported in allotype suppressed animals; a system potentially compromised by large numbers of T-suppressor cells.

In this communication we describe the use of thymectomized, lethally-irradiated, foetal liver reconstituted animals given graded numbers of T cells and immunized with a thymus-dependent antigen. The generation of memory B cells was then determined by cell transfer to carrier-primed irradiated recipients. Both the amount and the affinity of the primary and secondary antibody response were determined. Under these conditions we found that T cells were required for the generation of high-affinity memory B cells.

MATERIALS AND METHODS

Animals

All experiments employed female F_1 hybrid rats of the inbred strains Lewis and Brown Norway (LBN) and were obtained commercially (Microbiological Associates).

Reagents

Dinitrophenylated bovine gamma globulin (DNP-BGG) was prepared as previously described (Feldbush, 1973) and contained 40 mol DNP/mol of protein. [^{3}H]-DNP-lysine was obtained commercially (New England Nuclear Corporation) and checked for purity monthly by thin layer chromatography on silica gel using propanol: ammonium hydroxide (7:3 v/v) as developing solvents.

Immunization

Rats were immunized i.p. with 1·0 mg (0·1 ml) alum precipitated DNP-BGG plus 2×10^9 killed *Bordetella pertussis* organisms (Pertussis Vaccine, Wellcome, Ltd, Beckenham) as previously described (Feldbush, 1973).

Carrier primed recipients

Syngeneic LBN rats were primed with 1·0 mg (0·1 ml) alum-precipitated BGG plus 2×10^9 killed *B. pertussis* organisms injected i.p. Three weeks after priming rats were given 500 rad whole body *X*-irradiation and used as adoptive recipients 24 hr after irradiation.

Irradiation

Rats were placed in a lucite cage on a rotating table 60 cm from the *X*-irradiation source. *X*-ray was adminis-

tered from a 250 kVp 30mA *X*-ray machine with the beam filtered through 1·0 mm aluminium and 0·25 mm copper at a dose rate of 85 rad/min.

Purification of T cells

T cells were 'purified' by rosette formation and density centrifugation on Ficoll-Hypaque as previously described (Feldbush, 1980). Affinity column-purified rabbit anti-rat light chain or anti-immunoglobulin serum was coupled to sheep red blood cells (SRBC) with chromic chloride. Sensitized SRBC were incubated with cell suspensions of mesenteric and cervical lymph nodes from LBN rats at 4° for 45 min. Following incubation the cell suspension was overlaid on Ficoll-Hypaque (1·09 gm/cc) and centrifuged at 2200 ***g*** for 30 min at 25°. Interface cells (T cells) were collected and assayed for surface Ig by immunofluorescence. Only preparations of greater than 99% T cells were used.

Experimental design

LBN rats were thymectomized at 8 weeks of age according to the procedure of Lubaroff (personal communication). Two weeks post thymectomy rats were lethally irradiated (750 rad) and given at least 3×10^7 18-day foetal liver cells. After four weeks animals were given graded doses of purified T cells. One day after T-cell transfer, all animals were immunized with 1·0 mg DNP-BGG and bled on days +7, +14, +21 and +28. After the last bleed the animals were killed and cell suspensions prepared from the spleen and mesenteric lymph nodes. Cells (4×10^7) were then transferred to carrier-primed irradiated recipients to determine memory development. Adoptive recipients were challenged one day after cell transfer by injecting 1·0 mg DNP-BGG in saline intravenously (i.v.). At the time of killing, an autopsy was performed on all donor animals for detection of residual thymic tissue. Suspect tissue was examined histologically. Each experimental group consisted of three animals.

Antibody determination

Antibody to DNP-lysine was determined by the 50% ammonium sulphate method as previously described (Feldbush, 1973). The antigen-binding capacity (ABC) of the serum was calculated and expressed as nanograms of DNP-lysine bound per one ml of whole serum (Feldbush, 1973). 2-Me sensitive and resistant antibody titres were also determined using the haemagglutination assay. SRBC were sensitized with DNP-conjugated chicken anti-SRBC gamma globulin at a subagglutinating dilution. Fifty microlitres of test serum was diluted two-fold in 'u' bottom microtitre plates (Flow Laboratories, Rockville, Md) using a Titer-Tek Automatic Diluter (Microtiter, Cook Engineering, Alexandria, Va). Fifty microlitres of sensitized SRBC were added to each well and the antibody titre determined after overnight incubation. 2-Me resistant antibody titres were determined by the same method after test sera were incubated with an equal volume of 0·1 M 2-Me for 30 min at 37°.

Relative affinity determinations

The determination of the relative affinity of anti-DNP antibodies was performed using a modification of the Farr technique (Werblin *et al.*, 1973; Kim & Siskind, 1974; Kim, Kalver & Siskind, 1975). Serum samples from each experimental group were pooled and diluted to 20% serum with phosphate-buffered saline (PBS). These samples were then diluted to a constant antibody concentration of 0·01 μg ABC as determined by the Farr assay with 20% donor calf serum PBS to maintain a constant protein concentration. As carried out here 0·25 ml of varying dilutions of [^{3}H]-DNP-lysine were mixed with 0·25 ml of serum sample and kept overnight at 4°. Cold saturated ammonium sulphate (0·5 ml) was added and the tubes mixed and held for 30 min at 4°. The tubes were then centrifuged at 1200 ***g*** at 4° for 30 min.

The supernatants were decanted into separate tubes from which triplicate 0·1 ml samples were taken. The samples were mixed with 0·9 ml 0·1 N HCl and 9 ml triton toluene scintillation cocktail. Hapten input was determined by mixing 0·25 ml hapten dilution with 0·25 ml PBS for each hapten concentration. Non-specific binding was determined by mixing 0·25 ml hapten dilution with 0·25 ml 20% donor calf serum PBS for each hapten concentration. The data were plotted as AbH/H versus AbH (Pinckard & Weir, 1973) and the average association constants calculated at 25 (K_{25}), 50 (K_{50}) and 75 (K_{75}) percentage saturation of binding sites. Using this procedure small amounts of low and high affinity antibody are less likely to be missed (Kim & Siskind, 1974). Each serum pool was tested twice and the results averaged. Fifteen hapten concentrations, ranging from 2·5–1000 nM were used for each serum.

Evaluation of results

In order to establish what constituted a significant difference of relative affinity values, the reproduci-

bility of the assay was evaluated. For ease of data handling, the log of duplicate relative affinity values ($n = 120$) from these and other experiments was used to estimate the variance of the determination by $[(x_1 - x_2)^2]/2$. The mean of the variance at each level of saturation (25%, 50% and 75%) was averaged to yield the final estimate of variance for the assay. Based upon the normal distribution, a confidence interval can be constructed to evaluate relative affinity determinations by $x_1/x_2 \geq 10^{ts}$. The sample estimate of the variance of each test (S^2) computed above, was found to be 0·150. Each test mean was based on two observations. Therefore the standard deviation for the difference of these two means is: $S_{diff.} = [(S/\sqrt{S})\sqrt{2}] = S_{test} = 0{\cdot}150$. For the 95% confidence interval, the ratio of the relative affinity values (higher:lower) must be greater than 1·97 to be significant, and the ratio must exceed 2·54 for the 99% confidence interval.

In this communication we introduce the term maturation index (MI). This represents the ratio of the relative affinity of the adoptive secondary response to the relative affinity of the primary response. Based upon the preceding discussion of the reproducibility of the affinity assay, the maturation index must exceed a value of 2 in order to constitute a significant change in the affinity of the response.

RESULTS

Primary response with various numbers of T cells

To investigate T-cell involvement in the immune response, 'B' rats were given graded doses of purified T-cell lymphocytes 1 day before immunization with DNP-BGG plus *B. pertussis* (see Table 1). The primary response was monitored for 4 weeks after immunization. This was done so that T-cell requirements for the primary response could be compared to T-cell requirements for the generation of a memory response. Also, these measurements provided a method of determining when limited numbers of T cells were being reached.

The response elicited at each dose of T cells was tested at least twice. As can be seen in Table 1 (experiment A) the primary response increased as the numbers of T cells were increased. When T cells were injected in very low doses (experiment B) no relationship was observed between the amount of antibody produced and the T-cell concentration. However, at these low T-cell doses, only one animal in three developed a response higher than the control, non-T cell-reconstituted animals. This suggested that limiting numbers of T cells had been reached.

Memory cell development with various numbers of T cells

Pooled spleen and mesenteric lymph node cell preparations were made from the experimental groups of immunized 'B' rats given graded numbers of T cells. The cell preparations were transferred to 3-week carrier-primed irradiated recipients. All recipients were challenged 1 day after cell transfer and bled on day 7, 11 and 14 after challenge. The secondary

Table 1. Primary responses of B rats reconstituted with foetal liver and given graded numbers of T cells

	ABC* (day of bleeding)			
T cells (*n*)	7	14	21	28
Experiment A				
1×10^7	38±5†	1358±115	1036±38	622±17
5×10^6	0	720±173	560±98	416±21
$2{\cdot}5 \times 10^6$	0	586±43	425±46	210±28
0	0	330±269	300±245	148±121
Experiment B				
5×10^5	0	340±252	184±103	117±36
1×10^5	44±1	561±103	356±99	203±69
0	0	270±64	197±18	113±14

* ABC, antigen-binding capacity in ng/ml.
† The mean ABC is shown ± one standard deviation.

response was tested at least twice for each T-cell dose used and the results of one of these experiments are shown in Table 2. Preliminary experiments had shown that the peak response was never reached before day 7, thus earlier serum samples were not examined in these studies.

Two differences appeared in the T-cell dose–response relationships for the generation of memory cells versus the generation of antibody-forming cells in the primary response: (i) both the 1×10^5 and 5×10^5 T-cell doses resulted in significant memory B-cell formation (Table 2), whereas these doses of T cells resulted in marginal primary responses (Table 1); (ii) optimal memory-cell generation was produced by 5×10^6 T cells (Table 2) rather than 1×10^7 T cells which was optimum for the primary response. The latter T-cell dose seems to favour antibody-forming cell development over memory cell development. However, over a narrow range of T-cell concentrations, the memory response increased as the dose of T cells was increased. This is consistent with the concept of T cell requirement for memory cell generation.

Serum samples were also tested using the haemagglutination assay before and after 2-Me treatment. As can be seen in Table 3, 2-Me resistant antibody was produced in both the presence and absence of T cells, but was higher when adequate T-cell help was supplied. One point which should be noted is that a response was measured with the haemagglutination assay for animals which received no T cells (Table 3) whereas little or no response was detected using the Farr assay. One may conclude from these results that the Farr assay is substantially less sensitive in detecting IgM antibody compared with the haemagglutination assay. This is presumably an affinity-dependent phenomenon (Kim & Siskind, 1974), or related to the number of antibody-binding sites.

In previous experiments we have shown that complement receptor (CR) positive and negative memory cells can be transferred to carrier-primed recipients and display linear dose–response curves (Feldbush, 1980). While this implies that the adoptive transfer system is a valid and sensitive measure for the quantification of memory B cells, it does not negate a possible complicating role of helper T cells in the primed population. This is especially true if T cell help is limiting in the carrier-primed irradiated recipient. This possibility was examined by transferring purified DNP-BGG primed B cells to BGG-primed recipients, DNP-BGG primed T plus B cells to BGG-primed recipients and primed T and B cells to normal (non-primed) recipients. In all cases the antibody response was the same (ABC of 1·55, 1·49 and 1·47, respectively). This demonstrates that T-cell help in the carrier-primed recipient is sufficient to yield maximal antibody production by primed B cells, and that primed T cells, when transferred with primed B cells, have no enhancing effect upon the response.

Table 2. Secondary adoptive response of cells from B rats given graded numbers of T lymphocytes

T cells (n)	ABC* (day of bleeding) 7	11	14
Experiment A			
1×10^7	676±45†	513±58	344±44
5×10^6	972±48	744±17	577±16
$2{\cdot}5 \times 10^6$	633±108	363±58	248±45
0	270±54	175±7	111±10
Experiment B			
5×10^5	737±23	586±17	525±8
1×10^5	342±23	187±17	128±16
0	40±14	67±30	65±33

* ABC, antigen-binding capacity in ng/ml.
† The mean ABC is shown ± one standard deviation.

Table 3. Haemagglutination titres of secondary antibody response before and after 2-Me treatment

T cells (n)	Log_2 haemagglutinin titre (day of bleeding) 7	11	14
Experiment A			
1×10^7	9·3/8·3*	8·7/7·7	8·0/7
5×10^6	10·5/9	9·5/9	9·0/8·5
$2{\cdot}5 \times 10^6$	9·7/9	8·3/8·3	6·7/7·7
0	8·5/7	7·5/6	8·0/5
Experiment B			
5×10^5	8·5/7	8·0/7·5	8·0/7
1×10^5	7·0/6·5	6·5/5	6·0/5
0	4·75/2·25	5·5/2·5	4·0/2

* Mean of titre before 2-Me/mean of titre after 2-Me treatment.

Effect of graded doses of T cells on the quality of the immune response

Table 4 shows the association constants of the primary and secondary responses of B rats reconstituted with foetal liver and given graded doses of T cells. A comparison of the association constants of the primary response shows no significant difference in affinity at any level of T-cell reconstitution. Thus, when reconstituting with foetal liver cells, T cells do not exert selective pressure on subpopulations of B cells in the primary response. The ratio of the affinity of the secondary response to the affinity of the primary response has been termed the maturation index as discussed in Materials and Methods. From Table 4 it can be seen that in the absence of T cells, no maturation of the response was observed (MI < 2). In contrast, the presence of T cells allows for a significant maturation (MI > 2) of the response to occur. The maturation index also showed a tendency to increase with increasing numbers of T cells at least up to the 5×10^6 T cell-reconstitution dose. The slight drop in the maturation index at 1×10^7 T cells may be due to the recruitment of more cells into the primary response at the expense of the memory cell generation as was discussed above. The predominant effect of T cells was to increase the amount of high-affinity antibody in the secondary response which is reflected by the greatest maturation indexes being exhibited at K_{25}.

Primary and secondary responses of neonatal liver cells

Evidence has recently been presented showing differences in the heterogeneity of responses obtained with animals reconstituted with foetal, neonatal or adult B cells. Goidl & Siskind (1974) have shown that the adoptive primary response in animals reconstituted with foetal or neonatal cells was highly restricted with respect to heterogeneity or affinity as compared with adult spleen or bone marrow. The same restriction in heterogeneity of foetal and neonatal cells has also been shown for secondary responses to the antigens HGG and BGG (Szewczuk, Sherr, Cornacchia, Kim & Siskind, 1979). Studies on B-cell ontogeny have shown responses of normal heterogeneity developed shortly after birth (3–7 days; Sherr, Szewczuk & Siskind,

Table 4. Affinity of antibody produced in thymectomized, lethally-irradiated, foetal liver-reconstituted rats given graded numbers of purified T cells.

T cells (*n*)		K_{25}*	MI†	K_{50}	MI	K_{75}	MI
Experiment A							
0	1°‡	$5{\cdot}87 \times 10^8$		$1{\cdot}4 \times 10^8$		$4{\cdot}5 \times 10^7$	
	2°§	$1{\cdot}04 \times 10^9$	1·81	$2{\cdot}31 \times 10^8$	1·65	$6{\cdot}67 \times 10^7$	1·48
1×10^5	1°	$6{\cdot}97 \times 10^8$		$1{\cdot}4 \times 10^8$		$5{\cdot}06 \times 10^7$	
	2°	$2{\cdot}98 \times 10^9$	4·3	$5{\cdot}55 \times 10^8$	3·96	$9{\cdot}2 \times 10^7$	1·8
5×10^5	1°	$5{\cdot}9 \times 10^8$		$1{\cdot}29 \times 10^8$		$3{\cdot}4 \times 10^7$	
	2°	$2{\cdot}83 \times 10^9$	4·8	$4{\cdot}6 \times 10^8$	3·56	$1{\cdot}1 \times 10^8$	3·2
Experiment B							
0	1°	$5{\cdot}7 \times 10^8$		$1{\cdot}47 \times 10^8$		$3{\cdot}56 \times 10^7$	
	2°	$1{\cdot}03 \times 10^9$	1·81	$1{\cdot}48 \times 10^8$	1·0	$2{\cdot}7 \times 10^7$	0·76
$2{\cdot}5 \times 10^6$	1°	$4{\cdot}5 \times 10^8$		$9{\cdot}32 \times 10^7$		$2{\cdot}78 \times 10^7$	
	2°	$1{\cdot}29 \times 10^9$	2·87	$2{\cdot}17 \times 10^8$	2·33	$5{\cdot}6 \times 10^7$	2
5×10^6	1°	$5{\cdot}1 \times 10^8$		$1{\cdot}3 \times 10^8$		$4{\cdot}3 \times 10^7$	
	2°	$5{\cdot}33 \times 10^9$	10·5	$6{\cdot}89 \times 10^8$	5·3	$9{\cdot}6 \times 10^7$	2·2
1×10^7	1°	$6{\cdot}94 \times 10^8$		$1{\cdot}57 \times 10^8$		4×10^7	
	2°	$5{\cdot}78 \times 10^9$	8·3	$8{\cdot}9 \times 10^8$	5·67	$1{\cdot}26 \times 10^8$	3·15
Normal¶	1°	$1{\cdot}08 \times 10^9$		$1{\cdot}94 \times 10^8$		$4{\cdot}3 \times 10^7$	
	2°	$1{\cdot}3 \times 10^{10}$	12·0	$2{\cdot}72 \times 10^9$	14	$3{\cdot}5 \times 10^8$	8·14

* Affinity determination of 25% (K_{25}), 50% (K_{50}) and 75% (K_{75}) saturation of antibody-combining sites.

† Maturation index, explained under Material and Methods as affinity of secondary response divided by the affinity of primary response.

‡ Primary response.

§ Secondary response.

¶ Normal, normal animal (non-irradiated or reconstituted) used as a control.

1978). Furthermore, the appearance of clonotypes reactive to DNP has been shown to occur sequentially in the neonatal mouse (Klinman & Press, 1975a, b). The possibility existed that our previous findings concerning the T-cell requirement of memory cell generation might be different with a more mature B-cell population. To examine this question the experiments were repeated with thymectomized, lethally-irradiated animals reconstituted with neonatal liver cells. These experiments also provided the opportunity to normalize the data since the full range of T-cell doses was used in a single experiment.

As was observed with foetal liver-reconstituted animals, there was no definite dose–response relationship of the primary response (Table 5) at limiting numbers of T cells (1×10^5, 5×10^5). Higher doses of T cells gave a dose-dependent response with an almost normal response observed with 1×10^7 T cells.

Spleen and lymph node cells were adoptively transferred to carrier-primed recipients as before and all animals were challenged one day after transfer. The memory response increased as the number of T cells used to reconstitute the 'B' rats was increased (Table 5). In contrast to foetal liver-reconstituted animals, animals which had received 1×10^7 T cells generated the highest memory response which was comparable to that observed from normal animals which were lethally irradiated and reconstituted with neonatal liver.

Table 5. Primary and secondary response of B rats reconstituted with neonatal liver cells

T cells (*n*)	Peak ABC response*	
	Primary response	Secondary response
1×10^7	2418±768†	2024±305
2×10^6	1320±453	940±246
5×10^5	717±367	763±290
1×10^5	925±162	410±85
0	269±118	220±23
NI irr‡	2242±94	1587±181

* The peak antigen-binding capacity is shown for both the primary and adoptive secondary responses. The titres were produced on day 14 after primary immunization and day 11 after challenge.

† The mean ABC is shown±one standard deviation.

‡ Normal (non-thymectomized) lethally-irradiated rats reconstituted with foetal liver cells.

Effect of graded doses of T cells and neonatal liver reconstitution on the quality of the immune response

The affinity of the primary response following neo-

Table 6. Affinity of antibody produced by thymectomized, lethally-irradiated, neonatal liver-reconstituted rats given graded numbers of purified T cells

T cells (*n*)		Affinity					
		K_{25}*	MI†	K_{50}*	MI†	K_{75}*	MI†
1×10^7	1°‡	$7{\cdot}83 \times 10^8$		$1{\cdot}36 \times 10^8$		$2{\cdot}9 \times 10^7$	
	2°§	$1{\cdot}2 \times 10^{10}$	15·3	$1{\cdot}87 \times 10^9$	13·7	$3{\cdot}55 \times 10^8$	12·2
2×10^6	1°	$7{\cdot}37 \times 10^8$		$1{\cdot}5 \times 10^8$		$2{\cdot}9 \times 10^7$	
	2°	$5{\cdot}77 \times 10^9$	7·83	$7{\cdot}6 \times 10^8$	5·07	$1{\cdot}0 \times 10^8$	3·45
5×10^5	1°	$6{\cdot}45 \times 10^8$		$1{\cdot}69 \times 10^8$		$3{\cdot}99 \times 10^7$	
	2°	$3{\cdot}1 \times 10^9$	4·81	$5{\cdot}1 \times 10^8$	3·02	$7{\cdot}5 \times 10^7$	1·88
1×10^5	1°	$5{\cdot}34 \times 10^8$		$1{\cdot}14 \times 10^8$		$2{\cdot}27 \times 10^7$	
	2°	$7{\cdot}84 \times 10^8$	1·47	$9{\cdot}8 \times 10^7$	0·86	$2{\cdot}6 \times 10^7$	1·15
0	1°	$1{\cdot}95 \times 10^8$		$4{\cdot}8 \times 10^7$		$1{\cdot}49 \times 10^7$	
	2°	3×10^8	1·54	$7{\cdot}58 \times 10^7$	1·58	$2{\cdot}59 \times 10^7$	1·74
NIIRRN¶	1°	$8{\cdot}3 \times 10^8$		$1{\cdot}4 \times 10^8$		$3{\cdot}3 \times 10^7$	
	2°	$2{\cdot}5 \times 10^{10}$	30·2	$4{\cdot}1 \times 10^9$	29·3	$4{\cdot}6 \times 10^8$	13·9

* Affinity determination at 25% (K_{25}), 50% (K_{50}) and 75% (K_{75}) saturation of antibody combining sites.

† Maturation index.

‡ Primary response.

§ Secondary response.

¶ Non-thymectomized normal rats lethally-irradiated, neonatal-liver reconstituted.

natal liver reconstitution appears to increase slightly (K_{25} and K_{50}) with increasing numbers of T cells but the difference is small and not significant (Table 6). Examination of the affinities of the secondary responses and the maturation indexes shows a definite correlation of affinity increase with increasing numbers of T cells. These responses are comparable to the responses seen in normal animals (Table 4), and approach those seen in non-thymectomized, lethally-irradiated neonatal reconstituted animals (Table 6). These results and the results from foetal liver reconstituted animals demonstrate the T-cell requirement for the generation of high-affinity memory B cells.

DISCUSSION

The purpose of this investigation was to address the current controversy regarding the T-cell requirement for the generation of B-memory cells. On the basis of the evidence presented here we conclude: (i) that memory B cells, especially high-affinity memory cells, do not develop in appreciable numbers in the absence of T cells. (ii) The memory B cells which do develop in the absence of T cells can secrete a significant 2-Me sensitive antibody response (experiment B, Table 3) as compared with the almost exclusive IgG memory responses generated in the presence of T cells. (iii) It is apparent that fewer T cells are needed to develop memory cells than are required for the expression of a primary response.

In the present experiments it was observed that in the absence of T cells only minimal primary and secondary responses were elicited. This response was not a typical thymus-independent response in that both 2-Me sensitive and resistant antibodies were observed. It is possible that the IgG response can be attributed to the adjuvant *B. pertussis*. This adjuvant has been shown to elicit IgG production to a thymus-independent antigen (Kong & Morse, 1976). It is also possible that the adjuvant may act to replace T cells (Dresser, 1972) or selectively amplify the IgG precursors stimulated by residual T cells, an activity of *B. pertussis* which has also been reported (Torrigiani, 1972). However, the present results are in agreement with previous reports (Okamura *et al.*, 1976) where minimal low-affinity responses were observed in the absence of T cells.

Evaluation of the primary response obtained from 'B' animals reconstituted with graded doses of T cells has shown that limiting levels of T-cell reconstitution were probably reached (Table 1). Analysis of memory cell generation by adoptive transfer to carrier-primed recipients and challenge demonstrated that B memory-cell generation increased in the presence of increasing numbers of T cells (Table 2) up to a dose of 5×10^6 T cells. 'B' animals reconstituted with 1×10^7 T cells yielded the highest primary response but did not result in the greatest generation of B-memory cells. This may be due to a difference in the ability of foetal liver cells to develop precursors of B cells (Sherr, Szewczuk & Siskind, 1978) as compared with adult B-cell precursors or it may represent recruitment of more precursors into the primary response under conditions of this high T-cell dose. To distinguish between these two possibilities the experiments were repeated with neonatal liver reconstituted animals. Analysis of B memory-cell development by adoptive transfer again showed an increase of B-cell memory with increasing levels of T-cell reconstitution. Furthermore, the highest dose of T cells also yielded the highest memory response, comparable with that obtained from normal (non-thymectomized) rats which were lethally-irradiated and neonatal liver reconstituted. Thus, with a more mature population of B-cell precursors, a more consistent result was obtained.

The data presented here demonstrated that the increase in the memory response in the presence of T cells was due to the generation of high-affinity B-memory cells. In the absence of T cells, no appreciable maturation of the response was observed. In both foetal liver and neonatal liver-reconstituted animals the frequency of high-affinity memory cells increased with increasing numbers of T-cell reconstitution. In the primary response, no increase in affinity in relation to T-cell numbers was observed with foetal liver-reconstituted animals, but minor increases in affinity with increasing T-cell numbers were seen in neonatal liver-reconstituted animals.

Our results appear to be at odds with those of other investigators who have argued against T-cell involvement in the generation of B-memory cells (Diamantstein & Blitstein-Willinger, 1974; Schrader, 1975; Shinohara & Tada, 1974; Roelants & Askonas, 1972). However, some of these investigations utilized thymectomized, lethally-irradiated, and bone marrow-reconstituted animals which can be held suspect due to the possibility of T-cell contamination of the marrow population. Roelants & Askonas (1972) have described demonstrable levels of T cells in their thymus-deprived mice. This is especially relevant since in the

data presented here even minimal numbers of T cells allowed for the generation of significant memory responses. The results presented in the present paper have overcome this problem by using graded levels of T-cell reconstitution and showing that memory increases as a function of T-cell concentrations. A second consideration about B memory-cell generation in the absence of T cells is the role of adjuvant used in some of the experiments. As discussed above, *B. pertussis*, presumably due to its LPS component, has been shown to stimulate antibody responses to thymus-dependent antigens in the absence of T cells (Kong & Morse, 1976). Therefore, the possibility exists that the T-cell requirements were bypassed in the experiments of Schrader (1975) due to the presence of adjuvant and in the experiments of Shinohara & Tada (1974) by the use of the antigen DNP-Salmonella which acts as its own adjuvant due to the gram negative Salmonella organism. In the present experiments no response, primary or secondary, was observed if antigen was administered to 'B' animals in the absence of adjuvant (data not shown).

Our results confirm the observations of Okamura *et al.* (1976) who showed that only low-affinity memory cells were generated in the absence of T cells, but also extend these observations to demonstrate a dose–response effect of T-cell numbers on memory B-cell generation. Concerning the affinity of the primary response, Gershon & Paul (1971), using a Farr ratio technique, have shown the affinity of the response to increase with increasing numbers of T cells. This observation was confirmed and extended by DeKruyff & Siskind (1979), using a plaque inhibition assay. In these experiments the authors demonstrate that restricted T-cell help (low numbers of T-cell reconstitution) resulted in only direct plaque forming cells (PFC) of low affinity and restricted heterogeneity. An increase in the numbers of T cells allowed for indirect PFC formation and a highly-heterogeneous high-avidity response. On the contrary, Sanfilippo & Scott (1976) could not demonstrate a difference in the avidity of the responses of normal animals and animals which had been rendered tolerant to the protein carrier for the DNP-carrier immunogen using a PFC inhibition assay. The implications of the two latter findings must however be taken with reservation in light of the recent reports of hapten augmentable plaques (Schrater, Goidl, Thorbecke & Siskind, 1979) which shed doubt on the reliability of plaque inhibition as a measure of antibody affinity.

In the present study, the affinity of the antibody in the primary response in normal animals (Table 4) or in non-thymectomized reconstituted animals (Table 6) were higher than the affinity of the non-T cell reconstituted groups, but this difference was not always significant. Furthermore, thymectomized reconstituted animals given T cells showed no increase (foetal liver reconstituted) or a slight but insignificant increase (neonatal liver reconstituted) in affinity in the primary response. This is in agreement with the observations of Sanfilippo & Scott (1976). Also, if we consider only IgG secretion, our data would concur with the observations of DeKruyff & Siskind (1979), who reported that once indirect plaques were detected, only the magnitude but not the avidity of the response increased with increasing numbers of T-cell reconstitution. No direct reconciliation of the present studies and those reported by Gershon & Paul (1971) is readily apparent. The method of affinity determination used by Gershon & Paul (1971) is quite unique and a statement of a significant difference was not discussed.

We feel that the results presented here demonstrate the T-cell requirement for the generation of memory B cells, especially high-affinity B cells. Thus helper T cells allow for the efficient selection, triggering and proliferation of B-lymphocyte subpopulations.

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