THE RELATIONSHIP BETWEEN ANTIGENIC STRUCTURE AND THE REQUIREMENT FOR THYMUS-DERIVED CELLS IN THE IMMUNE RESPONSE*,‡

By MARC FELDMANN§, M.B., and ANTONY BASTEN||, D.Phil.

(From the Walter and Eliza Hall Institute of Medical Research, Post Office, The Royal Melbourne Hospital, Victoria 3050, Australia)

(Received for publication 16 March 1971)

The relationship between the thymus and immunological competence of some rodents has been well documented (1). Both neonatal thymectomy and adult thymectomy followed by irradiation and bone marrow protection result in a diminished capacity to mount cell-mediated immune reactions. In addition some humoral antibody responses, for example to antigens such as heterologous erythrocytes (2), serum proteins (3–5), and haptenic determinants are impaired. In contrast other antigens, for example MS_2 phage (6), *Escherischia coli* endotoxin (7), pneumococcal polysaccharide (8, 9), and polymerized flagellin (10) can stimulate relatively normal antibody production in thymectomized hosts, though frequently the range of antigen doses studied was limited. This paradox raises the problem of the role of thymus-derived (T)[1] lymphocytes in those responses dependent on an intact thymus. Miller and Mitchell (11) have shown that T cells themselves do not secrete antibody in the classical sense but collaborate with thymus-independent (B) lymphocytes which then become antibody-forming cells (AFC). The importance of the helper function of T cells in antibody production in carrier–hapten systems has also been stressed by Mitchison (12), Benacerraf, and his colleagues (13, 14) and Cheers et al.[2] However, this work does not clarify the difference between thymus-dependent and thymus-independent antibody responses.

The purpose of the present communication is to examine this question in an in vitro mouse model using antigens of various physical forms. The flagellar

* This work was supported by grants from the National Health and Medical Research Council, Canberra, Australia, the Australian Research Grants Commission, and the U.S. Public Health Service grant No. AI-0-3958 to Professor G. J. V. Nossal.

‡ This is publication number 1524 from The Walter and Eliza Hall Institute of Medical Research.

§ Supported by a National Health and Medical Research Council Postgraduate Fellowship.

|| Supported by a Queen Elizabeth II Fellowship.

[1] *Abbreviations used in this paper:* AFC, antibody-forming cell; ATXBM, adult thymectomized, lethally irradiated, and bone marrow protected; B, thymus independent, "bursal equivalent" derived; DNP-SRC, dinitrophenylated SRC; DRC, donkey red cells; DRC-DNP, dinitrophenylated DRC; DRC-MON, DRC conjugated with MON; Fab'-DNP, dinitrophenylated Fab'; MON, monomeric flagellin; POL, polymerized flagellin; POL-DNP, dinitrophenylated polymerized flagellin; SRC, sheep red cells; T, thymus derived, or thymus processed; XBM, sham thymectomized, lethally irradiated, and bone marrow protected.

[2] Cheers, C., J. C. S. Breitner, M. Little, and J. F. A. P. Miller. 1971. Co-operation between carrier reactive and hapten sensitive cells in vitro. *Nature (London)*. In press.

antigens of *Salmonella adelaide*, and the dinitrophenyl (DNP) determinant were chosen for this purpose. The results suggest that thymus independence is not a property of certain antigenic determinants, but depends instead on the physical form of the immunogen. This conclusion provides strong evidence of a role for T cells in antigen presentation.

Materials and Methods

Animals.—Mice of the highly inbred CBA/H/Wehi strain were used. Their origin and maintenance have been described previously (15).

Adult Thymectomy.—Thymectomy was performed in young adult female mice 4–7 wk old by the method of Miller (16). Sham-operated animals underwent the same procedure except that the thymus was left *in situ*.

Irradiation.—3–4 wk after operation thymectomized or sham-operated mice were exposed to total body irradiation in a Philips (RT 250) 250 Kev machine (Philips Electronic Instruments, Mount Vernon, N.Y.). The dose used was 750–800 rads to midpoint with maximum backscatter at 15 ma and half value layer of 0.8 mm Cu. Within 3 hr of irradiation all mice were injected with $3\text{-}5 \times 10^6$ syngeneic bone marrow cells. They were used for experimental purposes 4–8 wk postrestoration.

Before use of the spleen cells in culture, the mediastinum of the thymectomized mice was examined for evidence of thymus remnants. The spleens of mice with any suspicious areas were discarded from the spleen cell pool. The thymectomized mice will be designated ATXBM and the sham-operated controls XBM.

Thoracic Duct Cannulation.—The thoracic ducts of ATXBM mice were kindly cannulated by Dr. John Sprent, using the method of Miller and Mitchell (15). To ensure optimal depletion of lymphocytes, all mice received intravenous infusions of isotonic saline for the first 24 hr after cannulation. The fistulae were maintained for at least 48 hr, and the mice rested for 4–6 days before use as spleen cell donors.

Tissue Culture.—The method described by Feldmann and Diener (17) was used. A spleen cell suspension was placed in a glass tube sealed by a dialysis membrane and suspended from the stopper of an Erlenmeyer flask containing culture medium. Eagle's minimal essential medium with nonessential amino acids was obtained from Grand Island Biological Co., Grand Island, N.Y. (Catalogue No. F-15) and was supplemented with 5% fetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia). Batch number 614 was used in all experiments described here. The cultures were placed in a humidified incubator at 37°C, in a gas flow of 10% CO_2, 7% O_2 in N_2 for 3.7 days unless otherwise stated.

Cell Suspensions.—Mice were killed by cervical dislocation, the spleens removed aseptically, minced, and strained through an 80 gauge stainless steel sieve into cold culture medium. Cells were counted in a hemocytometer. The cell concentration used for culture was 1.5×10^7 viable cells/ml unless otherwise stated. Cell viability was assessed by the eosin dye exclusion method (18).

Treatment with Anti-θ-Serum.—AKR anti-C_3H anti-θ-serum was prepared according to the method of Reif and Allen (19). Cytotoxic activity was assayed as described elsewhere (20) using guinea pig serum absorbed with agarose (21). For experimental purposes, 3×10^8 spleen cells from XBM or drained ATXBM mice were incubated with 1 ml of undiluted serum at 37°C for 30 min. The cells were washed twice, and absorbed guinea pig serum added for a further incubation period of 30 min at 37°C. Residual cells were washed, counted, and cultured.

Antigens.—Polymerized flagellin (POL) of *Salmonella adelaide* was prepared by the method of Ada, Nossal, Pye, and Abbot (22). Monomeric flagellin (MON) was prepared by acid dissociation of the polymeric form just before use. In order to prevent the possibility of repoly-

merization of some of the MON in vitro, oxidized flagellin was prepared by the method of Parish (23). POL was dissociated, diluted in 0.1 M phosphate buffer, pH 7.5, and allowed to react with chloramine-T for 30 min at 25°C. Final concentration of the reaction mixture was 5 mg/ml MON and 1.2 mg/ml chloramine-T. The latter reagent was removed by dialysis against phosphate-buffered saline.

Sheep red cells (SRC) were collected into Alsever's solution and kept at 4°C before use. When required for in vitro stimulation they were washed three times in saline and resuspended in culture medium to the required concentration for use in vitro (usually 3×10^6 cells/ml). Donkey red cells (DRC) were obtained from Commonwealth Serum Laboratories, stored, and washed as described for SRC.

DRC were coated with MON using chromic chloride (24). 0.1 ml of 10 mg/ml MON and 0.1 ml of 1 mg/ml chromic chloride were added simultaneously to 0.1 ml of packed DRC, mixed thoroughly, and left at room temperature for 5 min. They were then washed and tested for satisfactory coating with MON by agglutination with rabbit anti-POL serum. This procedure was always carried out on the day of culture.

The DNP determinant was noncovalently bound to DRC by means of rabbit anti-DRC Fab′ fragments conjugated with DNP. The Fab′ fragments were substituted with an average of three DNP groups per mole Fab′, and 100 μg of dinitrophenylated Fab′ (Fab′-DNP) was used to coat 1 ml of 10% DRC. This was the optimal amount of Fab′-DNP to coat DRC for revealing anti-DNP plaque-forming cells.

DNP-polymerized flagellin (POL-DNP) was prepared by reacting POL (5 mg/ml) with sodium 2,4-dinitrobenzene sulfonate in 0.2 M sodium carbonate/bicarbonate buffer, pH 9.5 (25). For use as an immunogen in vitro, DNP-POL with an average of one DNP group per monomer unit was used. The same preparation was employed in all the experiments described here. Good primary anti-DNP responses may be elicited by this method, as described by Feldmann (25).

Enumeration of Antibody-Forming Cells.—Antibody-forming cells to flagellin were enumerated by the adherence colony method (26) using *Salmonella derby* (H antigen fg, 0 antigen 1, 4, 12) as the indicator strain for detecting AFC to flagellin from *S. adelaide* (H antigen fg, 0 antigen 35).

AFC to SRC and DRC were detected by the Cunningham and Szenberg (27) modification of the Jerne plaque technique (28). The number of AFC to DNP was enumerated by separate assay of 0.1 ml samples of the cultured cell suspension with SRC and with SRC coated with rabbit anti-SRC Fab′ conjugated with DNP (25, 29). Optimal sensitivity was achieved by prior titration of the coupling ratios of Fab′-DNP to red cells. By subtracting the number of AFC obtained with DNP-SRC from those produced by unconjugated SRC, the number of specific anti-DNP–AFC was derived. All specific anti-DNP–AFC measured by this method were inhibited by the presence of DNP-lysine at 10^{-4} M in the plaque-revealing system. The control studies verifying that a primary anti-DNP response may occur in vitro have been described in more detail elsewhere (25).

Statistics.—Calculations of the standard error of the mean, P values according to Student's t test or the nonparametric rank test were performed on an IBM 7044 computer.

RESULTS

Thymus Independence of the Antibody Response to Polymerized Flagellin In Vitro.—Experiments from several groups have suggested that a normal antibody response to POL can take place in mice depleted of T cells. Thus Martin (10) was unable to demonstrate a significant difference in POL titers between either neonatally thymectomized or ATXBM mice and the controls, although

a marked reduction in AFC to SRC was observed in the same animals. Similar results were obtained in mice pretreated with antilymphocyte serum (10), and in vitro with spleens from neonatally thymectomized donors (P. J. Russell. 1970. Unpublished observations). In these studies POL was used at the optimally immunogenic concentration. However, it is well known that the immune response in thymectomized mice to some antigens, for example SRC, is markedly dose dependent (30, 31), thymus dependency being demonstrable only at the lower end of the dose range.

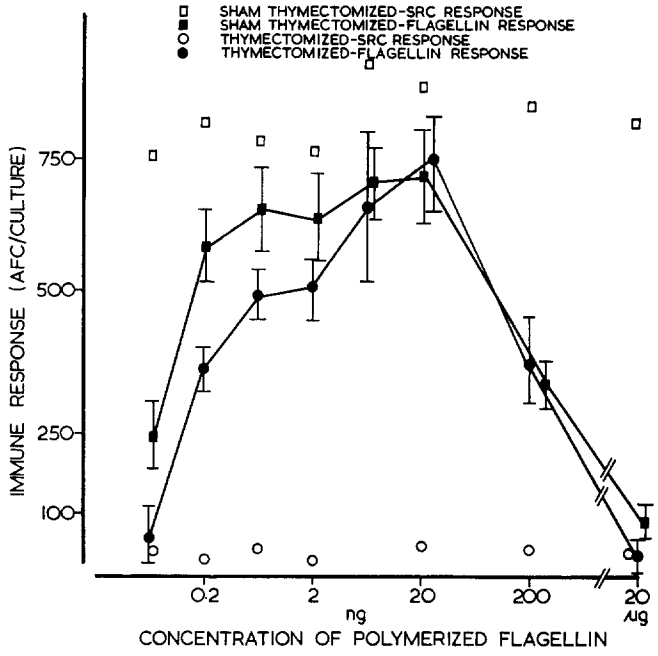

FIG. 1. The immune response of ATXBM and XBM cultures to POL. Each point represents the arithmetic mean of 10–20 cultures ± the standard error of the mean. The mean of the response of these cultures to SRC is indicated by the open symbols.

Three experiments were therefore performed to determine the response of thymectomized mice to different concentrations of POL in vitro. For this purpose spleen cells were obtained from ATXBM, XBM, and normal donors and cultured with POL and SRC. Cells from the irradiated mice were harvested 4–8 wk postirradiation at a time when XBM animals had recovered the capacity to respond to antigen in vivo. Two experiments measured AFC levels in ATXBM and XBM spleen cultures over the dose range 0.07 ng to 20 μg/ml POL,[3] while normal spleen cells were only cultured with 20 ng/ml POL. In the

[3] $1g = 10^3$ mg $= 10^6$ μg $= 10^9$ ng $= 10^{12}$ pg.

third experiment, the response of ATXBM spleen cells was compared with that of normal spleen cells to a similar range of POL concentration (0.07 ng to 20 μg/ml). The results of the three experiments were pooled and are summarized in Fig. 1. Antibody production by XBM and normal spleen cells was comparable; thus for clarity the latter group has been omitted from the graph. It can be seen that both thymectomized and nonthymectomized cultures responded similarly. Analysis of the pooled data by the nonparametic rank test and Student's t test failed to reveal a significant difference at any of the concentrations of POL used ($P>0.2$ in all cases). In contrast the same ATXBM cultures which responded to POL proved incapable of mounting an appreciable response to SRC, confirming that the SRC response is indeed thymus dependent, and that normal antibody production to POL can occur in a system significantly depleted of T cells by this functional criterion. The dichotomy in behavior of the two antigens demonstrated the specificity of the effect of POL.

Thymus Dependence of the Antibody Response to Monomeric Flagellin In Vitro.—One of the advantages of the flagellar antigens of *Salmonella adelaide* is the existence of three molecular forms. Each displays all the antigenic determinants of flagellin, but differs greatly in molecular weight and configuration from the others. This variation in antigenic form has already been utilized in studying the process of tolerance induction in vitro (17, 32). POL has a quaternary structure similar to that of bacterial flagella (33) which are composed of a number of monomeric units arranged in helices with 8–10 units per fibril of the helix. This provides a linear array of repeating antigenic determinants. MON, on the other hand, has a lower molecular weight (40,000) and can be split by cyanogen bromide into four fragments one of which, fragment A, contains all the antigenic determinants of flagellin (23).

It is possible that the thymus independence of POL is related to its quaternary structure. To test this hypothesis, the effect of thymectomy on antibody production to MON was examined. Spleen cells from ATXBM, XBM, and normal donors were cultured with various concentrations of oxidized MON (see Materials and Methods) and SRC as the control immunogen. The results of two such experiments are illustrated in Fig. 2. It can be seen that ATXBM spleens responded poorly to MON compared with the XBM and normal controls. This difference was highly significant ($P < 0.01$, Student's t test) at those concentrations of flagellin (10, 100, 1000 ng/ml) with which reasonable responses were obtained. The completeness of the thymectomy was verified by the low response of ATXBM cultures to SRC.

The Antibody Response to DRC Conjugated with Mon (DRC-MON) In Vitro.—The foregoing results suggested that the reason for the thymus independence of POL resided in its three-dimensional structure, and not in peculiarities of its antigenic determinants. This possibility was examined in another way, by

measuring the anti-flagellin response to MON coupled to DRC (see Materials and Methods). 10^6 or 10^7 DRC-MON cells proved approximately as immunogenic as 20 ng POL/ml, the optimal concentration of POL in vitro. To assess the thymus dependency of this form of challenge, ATXBM, XBM, and normal mouse spleens were cultured with 10^6–10^9 DRC-MON, and 3×10^6 SRC. Fig. 3 depicts the pooled data from three such experiments, and illustrates clearly that the response to flagellin administered to ATXBM cells as MON-

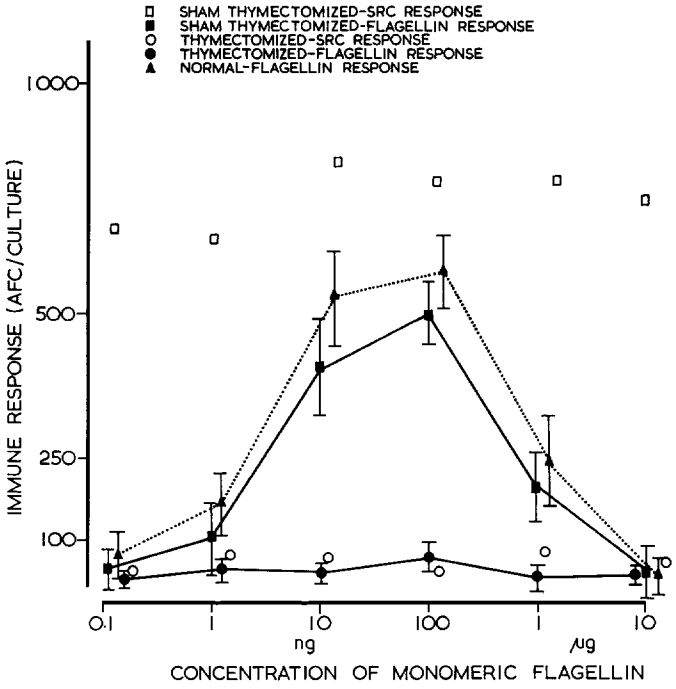

FIG. 2. The immune response of ATXBM, XBM, and normal spleen cultures to MON. Each point represents the arithmetic mean ± the standard error of the mean of 8–16 cultures. The mean of the response to SRC is indicated by the open symbols.

coated DRC was as thymus dependent as the response to the red cells themselves, over the dose range 10^6–10^8 red cells/ml ($P < 0.01$ for XBM *versus* ATXBM spleen cells). A higher number of red cells of any kind was toxic in vitro, as shown by the poor response to a normally highly immunogenic stimulus of 3×10^6 SRC/ml in the presence of 10^9 DRC-MON/ml. Cells from the same pool of ATXBM spleens produced normal numbers of AFC upon challenge with POL, confirming that the cultures could react with the antigenic determinants of flagellin. The response of ATXBM cells to DRC-MON and to POL differed significantly ($P < 0.01$).

The Antibody Response to POL-DNP and DRC-DNP In Vitro.—The data presented above implied that a critical factor in determining whether antibody production is thymus dependent or independent is the manner in which antigenic determinants are arranged, i.e., presented to lymphoid cells. The problem was further analyzed by comparing the responses to a haptenic determinant

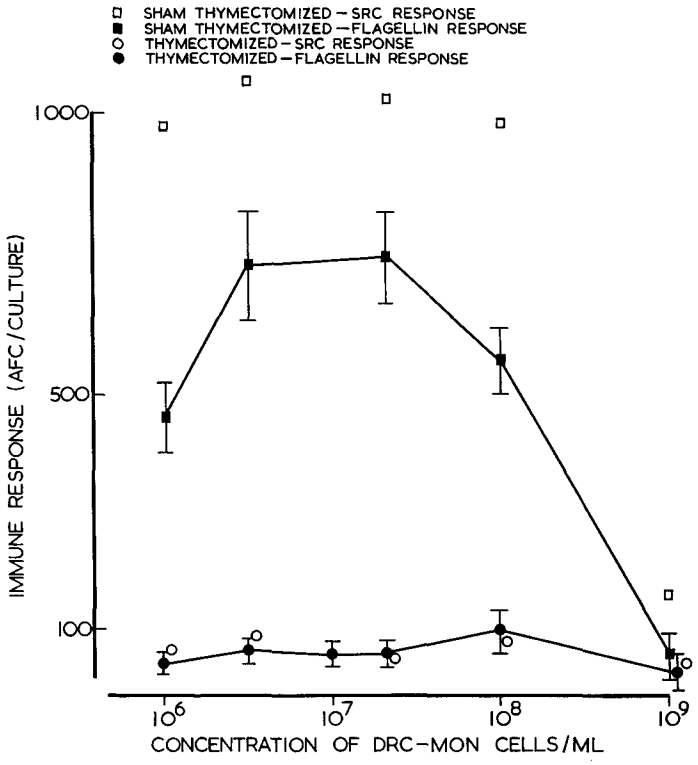

FIG. 3. The immune response of ATXBM and XBM cultures to DRC-MON. Each point represents the arithmetic mean of 12–18 cultures ± the standard error of the mean. The mean of the response to SRC is indicated by the open symbols.

(DNP) when it was conjugated to the two different carriers, POL and DRC. Fig. 4 compares the number of anti-DNP AFC obtained from ATXBM, XBM, and normal spleen cells after culture with various concentrations of POL-DNP (1 ng to 10 μg/ml). The results represent data pooled from four experiments, two of which incorporated the full dose range of POL-DNP. It can be seen that ATXBM spleen cells responded as well as the two control groups at all POL-DNP doses. The functional lack of T cells in ATXBM cultures was verified by the poor response to unconjugated DRC.

A marked contrast in anti-DNP antibody production was observed when the hapten was presented on a thymus-dependent carrier, DRC coated with DNP. Preliminary investigations indicated that the optimal dose of dinitrophenylated DRC (DRC-DNP) in vitro was 10^6–10^8 DNP-coated cells/ml which gave as many AFC as POL-DNP. Thus the antibody response of ATXBM, XBM, and normal spleen cells to 10^6–10^8 DRC-DNP cells was measured. Cul-

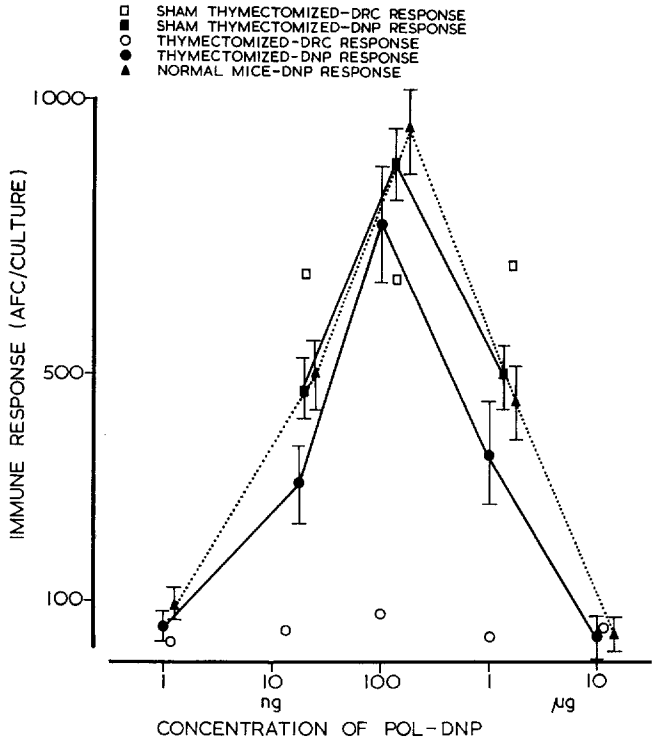

FIG. 4. The immune response of ATXBM, XBM, and normal spleen cultures to POL-DNP. Each point represents the arithmetic mean of 12–16 cultures ± the standard error of the mean. The mean of the response to DRC is indicated by the open symbols.

tures of cells from thymectomized donors, but not from XBM controls, responded poorly to DNP administered in this form, as shown in Fig. 5 ($P < 0.05$ at all the doses used). The anti-DNP response of the same ATXBM spleens to POL-DNP was about 6–10-fold greater ranging from means of 560 to 980 AFC/culture in the three experiments performed ($P < 0.05$). Although 10^8 DRC-DNP/ml produced more AFC than lower doses in two experiments out of three, the difference was not statistically significant ($0.05 < P < 0.1$).

Response of ATXBM Spleen Cells Further Depleted of T Cells by Chronic

Thoracic Duct Drainage and Anti-θ-Serum.—Spleens from ATXBM mice contain a small number of residual T cells (34). It was thus conceivable that the apparent thymus independence of POL or POL-DNP was due to an increased efficiency of T and B cell collaboration, facilitated in some way by the repeating determinants on these antigens. Experiments were thus performed in which ATXBM spleens were further depleted of their minor T cell component by using a combination of thoracic duct drainage and pretreatment with

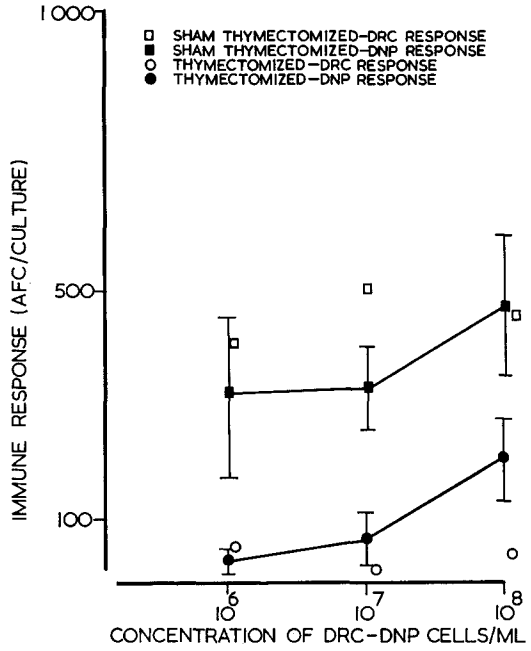

FIG. 5. The immune response of ATXBM and XBM cultures to DRC-DNP. Each point represents the arithmetic mean of 8 cultures ± the standard error of the mean. The mean of the response to DRC is indicated by the open symbols.

anti-θ-serum and complement (1, 34). The results obtained are shown in Table I, and indicate that no depression of the response to POL-DNP occurred despite the use of both procedures. In contrast, the usual very poor response to DRC in ATXBM cells was abolished by this means. The efficiency of the anti-θ-serum on T cells was confirmed by assay of its cytotoxic effect (eosin uptake) and also by the marked depression of the response of anti-θ-treated XBM mice to DRC, but not to POL-DNP. The fact that there was no effect of anti-θ-serum and drainage on the response to POL-DNP was very strong evidence that the response was truly thymus independent, i.e., that POL-DNP was capable of immunizing B cells directly.

The Antibody Response of ATXBM and XBM Spleen Cells at Various Cell densities.—The cellular composition of ATXBM and XBM spleens is not the same. Raff and Wortis (34), using the θ-antigen as a marker for T cells, found that 30–50% of normal spleen cells were thymus derived. In contrast only 3–5% of ATXBM spleen cells were θ-positive. Thus the relative content of antibody-forming cell precursors (B cells) is higher in ATXBM spleens. This extrapolation was recently verified indirectly by Raff with a rabbit anti-mouse anti-B cell serum (35). Thus there may be some difficulty in comparing the response to POL or POL-DNP in cultures of XBM and ATXBM spleens which contain a standard number of viable cells (1.5×10^7 per culture). Conceivably the thymus independence of these responses may have been due to either a greater number of B cells per culture or an alteration in the T to B cell ratio. To exclude these possibilities, different numbers of ATXBM and XBM cells were cultured together with SRC and either POL or MON. Table II presents the results of this experiment. At no spleen dose did the ATXBM cells respond significantly to SRC, while AFC levels in culture containing the highest ATXBM spleen cell dose (25 and 30×10^6) were not enhanced. Nor was the response of 10^7 ATXBM cells to POL much less than that of 10^7 or 1.5×10^7 XBM cells. These results did not support the possibility that the differences observed were due to variations in the number of B cells in each kind of spleen cell population.

TABLE I

Effect of Further T Cell Depletion on the Immune Response of ATXBM Spleen Cells to POL-DNP and DRC

	Cell source	Treatment	Immune response (AFC/culture ± SE)	
			POL-DNP	DRC
A	ATXBM	Nil	1040 ± 80	134 ± 48
B	Cannulated ATXBM	Nil	1180 ± 180	90 ± 30
C	"	Anti-θ and complement	1060 ± 90	22 ± 10*
D	XBM	Nil	960 ± 120	2060 ± 320
E	"	Anti-θ and complement	1220 ± 170	310 ± 160

The data were pooled from three experiments. Each value represents the arithmetic mean of 12 cultures ± the standard error of the mean, except for group B which consisted of 4 cultures. There was no difference between the immune responses to DNP in any of the groups.

By contrast, the immune response of XBM cultures to DRC was significantly diminished ($P < 0.01$).

* The response of ATXBM cultures to DRC was lowered by anti-θ–treatment ($P < 0.05$).

Response of ATXBM and XBM Spleen Cells after Various Times in Culture.—It was conceivable that the lowered response of spleen cells from ATXBM

TABLE II

The Immune Response of ATXBM and XBM Spleen Cells Cultivated at Various Cell Densities

Cell source	Number ($\times 10^6$)	Immune response (AFC/culture ± SE)	
		SRC	POL
ATXBM	5	<50	20 ± 40
	10	<50	480 ± 160
	15	225 ± 125	820 ± 120
	25	420 ± 200	860 ± 140
	30	100 ± 60	320 ± 200
XBM	5	250 ± 80	80 ± 40
	10	1080 ± 420	360 ± 80
	15	1600 ± 280	680 ± 70
	25	1860 ± 200	480 ± 180
	30	1105 ± 225	180 ± 60

The results represent the arithmetic means ± the standard error of the mean of four cultures. The responses of the XBM and ATXBM cells to SRC were significantly different at all cell doses ($P < 0.05$), whereas the responses to POL were the same at comparable cell doses.

TABLE III

The Kinetics of the Response of ATXBM and XBM Spleen Cell Cultures to SRC and POL
(AFC/Culture ± SE)

Period of culture	Immune response of			
	XBM cells		ATXBM cells	
	SRC	POL	SRC	POL
(*days*)				
2.7	1680 ± 320	480 ± 105	140 ± 80	320 ± 30
3.7	2440 ± 280	720 ± 150	180 ± 90	640 ± 280
4.7	2060 ± 140	640 ± 280	205 ± 80	600 ± 210
5.7	1040 ± 300	360 ± 140	80 ± 80	360 ± 120
6.7	520 ± 380	120 ± 140	<50	100 ± 80

The results shown are from a typical experiment. Each point represents the arithmetic mean of four cultures ± the standard error of the mean. The difference between the response to SRC of thymectomized and sham-thymectomized cultures was significant at all the time points tested ($P < 0.01$), whereas there was no difference in the response to POL.

mice to MON or SRC, compared with those from XBM donors, may have been due to different kinetics, i.e., antibody production by ATXBM cells may not have reached optimal levels at 3.7 days of culture. To test this possibility, ATXBM and XBM cultures immunized with SRC and POL were harvested at 2.7, 3.7, 4.7, 5.7, and 6.7 days. The ratio of the response of XBM:ATXBM cultures was not lowered at these other times (Table III).

DISCUSSION

The role of T cells in cell-mediated immunity is now well defined, but their precise task in humoral immunity is less clear. Thus antibody production to certain antigens such as heterologous erythrocytes, serum proteins, and hapten–protein conjugates (1) requires the presence of T cells in a helper capacity, while responses to other antigens, for example MS_2 phage (6) or POL (10) are apparently thymus independent. Various theories have been proposed to account for this difference in T cell requirements. For example, several investigators have suggested that the effect of neonatal thymectomy depends on the time at which new born animals become capable of responding to a particular antigen. In other words, if sufficient thymus influence has been exerted early in life, by day 1 in the case of MS_2 phage, then neonatal thymectomy should not have much effect on antibody production to that antigen. If this explanation is correct, it might be predicted that the response of ATXBM mice, in which thymus influence has been destroyed by thymectomy and irradiation, would be minimal. However, prior immunization of such animals has been shown to result in some residual capacity to produce antibody after irradiation (36). Therefore relatively normal responses in ATXBM mice could be due to previous stimulation with cross-reactive antigenic determinants. Maturation hypotheses of this kind are determinant specific and suggest that the reason for thymus dependency is related to the nature of the determinants in question.

Alternatively the T cell requirements for a particular antibody response might depend on the way antigen is presented to the host, as suggested by Mitchison (37) and confirmed by the investigations reported here. The flagellar antigens were selected for this study because they could be readily obtained in various physical forms, each of which contains the same antigenic determinants. It was found that spleen cells from ATXBM mice responded well in vitro to polymerized, but not to monomeric flagellin or to flagellin-coated red cells, whereas antibody production by XBM and normal spleens to all physical forms was comparable (Figs. 1–3). The dichotomy in the responses of the same ATXBM spleen cells to SRC and POL indicated that normal antibody production to POL could take place despite a significant deficit in T cells. As a corollary to this point, the thymus independence of POL was shown to be antigen specific. In other words the possibility that POL might have some nonspecific stimulatory or adjuvant effect on spleen cells from ATXBM mice was excluded. The capacity of POL to immunize ATXBM cultures did not depend on the amount of antigen used (Fig. 1). Thus POL differed in this respect from some other antigens, for example SRC, which can elicit normal responses in thymectomized mice at high, but not at low doses (30, 31). The demonstration of comparable AFC levels to POL in cultures of ATXBM, XBM, and normal spleens implied a similar mechanism of immune induction in each case.

In contrast the in vitro response to the monomeric form of flagellin was thymus dependent at all doses of antigen used (Fig. 2). This finding may have been due in part to the restriction on maximum concentration of antigen which can be utilized in vitro. Doses of MON exceeding 10 μg/ml are known to be nonimmunogenic even when cultured with normal spleen cells (17). For this reason the thymus dependency of MON was tested in another way by coupling flagellin to a carrier such as DRC which itself was thymus dependent. Satisfactory coating of the red cells was verified by agglutination with rabbit anti-POL serum. The results (Fig. 3) were in one way less easy to interpret, as antibody formation to MON in this form becomes macrophage dependent (Feldmann, M. To be published). Nevertheless it is apparent from the data in Fig. 3 that the response to flagellin presented as MON-DRC did not occur in the absence of T cells.

The experiments described above suggest that the requirement for T cells in the immune response to flagellar antigens depends on the way in which the antigenic determinants are presented. It was therefore important to establish whether this was a general phenomenon or merely restricted to flagellin. The approach used was to compare the responses of cultures of spleen cells from ATXBM mice to two different forms of the DNP determinant, either on the thymus-independent carrier, POL or on the thymus dependent carrier, DRC. If the conclusions reached with flagellin were valid, then a specific anti-DNP response should occur in cultures of ATXBM spleen cells immunized with DNP-POL but not with DNP-DRC. As shown in Fig. 4–5, this prediction proved correct. Control cultures of XBM and normal spleen cells responded equally well to both immunogenic forms.

Similar observations have been made by J. Palmer and W. Byrd (unpublished observations). These workers found that the immune response of neonatally thymectomized mice to intact SRC in vitro was greatly reduced, while that to soluble SRC antigen was normal. We have confirmed their observations in ATXBM cultures (unpublished data) and the results strongly support the contention that thymus dependency is related to the mode of presentation of the antigenic determinants rather than to their specificity.

In drawing such a conclusion the assumption was made that spleens from ATXBM mice contained an insignificant number of functional T cells. However, treatment of similar spleens with anti-θ-serum has revealed that 3–5% of the lymphocytes were killed, and were thus presumably T cells (34). These T cells could have originated either from the hemopoietic stem cells used for host protection after irradiation or from remnants of the pool of recirculating lymphocytes. Whatever the source it could be argued that certain antigens such as POL are not truly thymus independent but merely require very small numbers of T cells for collaboration with B cells.

An attempt to answer this criticism was made by applying a combination of

methods of T cell depletion to ATXBM mice, each of which on its own has been shown to diminish antibody production significantly (1, 38). The failure of both chronic thoracic duct drainage and treatment with anti-θ-serum and complement (Table I) to reduce the response of ATXBM spleen cells to POL-DNP confirmed that antigenic determinants presented in an appropriate form can immunize B cells directly.

Proliferation of T cells has been shown to be one of the consequences of antigenic stimulation (9). Immunization of neonatally thymectomized or ATXBM mice might therefore result in multiplication of any residual T cells with restoration of the antibody response. A mechanism of this kind probably accounts for the effectiveness of polyadenylic–polyuridylic acid in thymectomized animals (39). However, this explanation is unlikely to apply to the experiments described here. Davies et al. (9) for example, have demonstrated that flagella from *Salmonella typhi* were poor stimulators of T cell proliferation. In addition, two of our findings were inconsistent with this concept: First, depletion of residual T cells in the spleens of ATXBM mice by two further means did not impair their response to antigens on POL. Secondly, no difference between the responses of ATXBM and XBM spleen cells to POL or POL-DNP was observed, even as early as 2.7 days after initiation of culture. This would leave little time for substantial proliferation of T cells before interaction with B cells took place.

The experiments described above shed some light on the mechanism of T and B cell collaboration in the antibody response. The ability of the polymeric form of flagellin or DNP-POL to trigger B cells directly suggests that the role of the T cell is the presentation of antigen to AFCP (B cells) in such a way as to initiate the differentiation sequence of antibody synthesis. Thus the distribution of antigenic determinants on POL may mimic the pattern of antigen on the surface of antigen-activated T cells. It is of interest in this context that other thymus-independent antigens, *Escherichia coli* endotoxin, and polyvinyl pyrrolidone (See reference 7) also possess repeating antigenic determinants. These experiments do not clarify whether antigen presentation by T cells is a passive process or an active one requiring proliferation on the part of the T cells. Alternatively the results can be interpreted in the light of the antigen concentration hypothesis, in which it is postulated that T cells not only have a specific receptor on their surface but can be activated by the corresponding antigenic determinants to synthesize and export the receptor in the form on an antibody-like molecule (IgX), thereby permitting concentration of antigen onto B cells. Conceivably the surface of a POL molecule could be sufficiently similar to that of the IgX–antigen complex to stimulate B cells directly.

SUMMARY

Certain antigens such as polymerized flagellin are capable of producing relatively normal antibody levels in thymectomized mice, whereas others, including

heterologous erythrocytes require the presence of T cells in a helper capacity. The mechanism of thymus-independent antibody production was investigated by comparing the primary IgM responses of spleen cells from ATXBM, XBM, and normal mice to various physical forms of the flagellar antigens of *Salmonella adelaide* in vitro. No reduction in antibody-forming cell levels to polymerized flagellin over a wide dose range was observed in ATXBM cultures, although the same spleen cells did not respond to an optimal dose of sheep red cells. In contrast, when flagellar determinants were presented in a monomeric form or as flagellin-coated donkey red cells, a highly significant difference was observed between the antibody responses of spleen cells from ATXBM mice and XBM or normal controls. The results suggested that the requirement for T cells in antibody production is not a property of specific antigenic determinants, but depends on the mode of antigenic presentation.

The validity of this conclusion was confirmed by using another antigenic determinant (DNP) coupled either to the thymus-independent carrier, POL, or to the thymus-dependent carrier, DRC. Spleen cells from XBM mice produced comparable AFC levels to both forms of DNP, but the results from ATXBM cultures showed a marked difference. The anti-DNP response to DNP-DRC was greatly reduced compared to controls, whereas that to DNP-POL was normal even after prolonged thoracic duct drainage of the ATXBM donors and pretreatment of their spleen cells with anti-θ-serum and complement. The data presented here imply that the role of T cells in humoral immunity is the presentation of antigen to B cells in such a manner as to initiate optimal antibody synthesis.

The authors wish to thank Mr. J. Pye for preparing the flagellin used in this study, Dr. John Sprent for cannulating ATXBM mice, and Mrs. J. Thompson and Miss G. Horlock for their excellent technical assistance.

BIBLIOGRAPHY

1. Miller, J. F. A. P., and D. Osoba. 1967. Current concepts of the immunological function of the thymus. *Physiol. Rev.* **47**:437.
2. Miller, J. F. A. P., P. M. DeBurgh, and G. A. Grant. 1965. Thymus and the production of antibody-plaque forming cells. *Nature* (*London*) **208**:1332.
3. Jankovic, B. D., B. H. Waksman, and B. G. Arnason. 1962. Role of thymus in immune reactions in rats. I. The immunologic response to bovine serum albumin (antibody formation, Arthus reactivity, and delayed hypersensitivity) in rats thymectomized or splenectomized at various times after birth. *J. Exp. Med.* **116**:159.
4. Good, R. A., A. P. Dalmasso, C. Martinez, O. K. Archer, J. C. Pierce, and B. W. Papermaster. 1962. The role of the thymus in development of immunologic capacity in rabbits and mice. *J. Exp. Med.* **116**:773.
5. Taylor, R. B. 1969. Cellular co-operation in the antibody response of mice to two serum albumins: specific function of thymus cells. *Transplant. Rev.* **1**:114.

6. Basch, R. S. 1966. Immunologic competence after thymectomy. *Int. Arch. Allergy Appl. Immunol.* **30:**105.
7. Anderson, B., and G. Moller. 1970. Observations quoted by O. Syoberg and E. Moller. Antigen binding cells in tolerant animals. *Nature (London).* **228:**780.
8. Humphrey, J. H., D. M. V. Parrott, and J. East. 1964. Studies in globulin and antibody production on mice thymectomized at birth. *Immunology.* **7:**419.
9. Davies, A. J. S., R. L. Carter, E. Leuchars, V. Wallis, and F. M. Dietrich. 1970. The morphology of immune reactions in normal, thymectomized and reconstituted mice. III. Response to bacterial antigens: salmonellar flagellar antigen and pneumococcal polysaccharide. *Immunology.* **19:**945.
10. Martin, W. J. 1969. Mode of action of antilymphocyte serum on thymus derived lymphocytes. Doctorate Thesis. University of Melbourne, Melbourne, Australia.
11. Miller, J. F. A. P., and G. F. Mitchell. 1969. Thymus and antigen reactive cells. *Transplant. Rev.* **1:**3.
12. Mitchison, N. A. The carrier effect on the secondary response to hapten protein conjugates. II. Cellular co-operation. *Eur. J. Immunol.* In press.
13. Katz, D. H., W. E. Paul, E. A. Goidl, and B. Benacerraf. 1970. Carrier function in anti-hapten immune responses. I. Enhancement of primary and secondary anti-hapten antibody responses by carrier preimmunization. *J. Exp. Med.* **132:**261.
14. Paul, W. E., D. H. Katz, E. A. Goidl, and B. Benacerraf. 1970. Carrier function in anti-hapten immune responses. II. Specific properties of carrier cells capable of enhancing anti-hapten antibody responses. *J. Exp. Med.* **132:**283.
15. Miller, J. F. A. P., and G. F. Mitchell. 1968. Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. *J. Exp. Med.* **128:**801.
16. Miller, J. F. A. P. 1960. Studies on mouse leukaemia. The role of thymus in leukaemogenesis by cell-free leukaemic filtrates. *Brit. J. Cancer.* **10:**431.
17. Feldmann, M., and E. Diener. 1971. Antibody mediated suppression of the immune response in vitro. III. Low zone tolerance in vitro. *Immunology.* In press.
18. Hanks, J. H., and J. H. Wallace. 1950. Determination of cell viability. *Proc. Soc. Exp. Biol. Med.* **98:**188.
19. Reif, A. E., and J. M. V. Allen. 1964. The AKR thymic antigen and its distribution in leukemias and nervous tissue. *J. Exp. Med.* **120:**413.
20. Miller, J. F. A. P., and J. Sprent. 1971. Thymus derived cells in mouse thoracic duct lymph. *Nature (London).* **230:**267.
21. Cohen, A., and M. Schlesinger. 1970. Absorption of guinea pig serum with agar. A method for elimination of its cytotoxicity for murine thymus cells. *Transplantation.* **10:**130.
22. Ada, G. L., G. J. V. Nossal, J. Pye, and A. Abbot. 1964. Antigens in immunity. I. Preparation and properties of flagellar antigen from Salmonella adelaide. *Aust. J. Exp. Biol. Med. Sci.* **42:**267.
23. Parish, C. R. 1969. Immunochemical studies of bacterial flagellin. Doctorate Thesis. University of Melbourne, Melbourne, Australia.
24. Perruca, P. J., W. P. Faulk, and H. Fudenberg. 1969. *J. Immunol.* **102:**812.

25. Feldmann, M. 1971. Induction of immunity and tolerance to the dinitrophenyl determinant in vitro. *Nature (London)*. In press.
26. Diener, E. 1968. A new method for the enumeration of single antibody producing cells. *J. Immunol.* **100:**1062.
27. Cunningham, A. J., and A. Szenberg. 1968. Further improvements on the plaque technique for detecting single antibody forming cells. *Immunology*. **14:**599.
28. Jerne, N. K., and A. A. Nordin. 1963. Plaque formation in agar by single antibody forming cells. *Science (Washington)*. **140:**405.
29. Strausbach, P., A. Sulica, and D. Givol. 1970. General method for the detection of cells producing antibodies to haptens and proteins. *Nature (London)*. **227:**68.
30. Sinclair, N. R. St. C., and E. V. Elliott. 1968. Neonatal thymectomy and the decrease in antigen sensitivity of the primary response and immunological memory systems. *Immunology*. **15:**325.
31. Taylor, R. B., and H. H. Wortis. 1968. Thymus dependence of antibody response: variation with dose of antigen and class of antibody. *Nature (London)*. **220:**927.
32. Diener, E., and M. Feldmann. 1970. Antibody-mediated suppression of the immune response in vitro. II. A new approach to the phenomenon of immunological tolerance. *J. Exp. Med.* **132:**31.
33. Lowy, J., and J. Hanson. 1965. Electron microscopic studies of bacterial flagella. *J. Mol. Biol.* **11:**293.
34. Raff, M. C., and H. Wortis. 1970. Thymus dependence of θ bearing cells in the peripheral lymphoid tissue of mice. *Immunology*. **18:**931.
35. Raff, M. C., S. Nase, and N. A. Mitchison. 1971. Mouse specific bone marrow derived lymphocyte antigen. A marker for thymus independent lymphocytes. *Nature (London)*. **229:**50.
36. Cross, A. M., E. Leuchars, and A. J. S. Davies. 1964. The retention of immunological information by thymectomized irradiated mice. II. By thymectomized syngeneic radiation chimaeras. *Nature (London)*. **203:**1042.
37. Mitchison, N. A. Cell co-operation in the immune response: the hypothesis of an antigen presentation mechanism. *Immunopathol. Int. Symp.* 6th. In press.
38. Schlesinger, M. 1970. Anti θ antibodies for detecting thymus-dependent lymphocytes in the immune response of mice to SRBC. *Nature (London)*. **226:**1255.
39. Cone, R. E. 1970. The amplification of thymus-influenced antigen reactive cells by complexes of polyadenylic–polyuridylic acids. Doctorate Thesis. University of Michigan, Ann Arbor, Michigan.