

## MATURATION OF THE HUMORAL IMMUNE RESPONSE IN MICE\*

BY PATRICIA G. SPEAR† AND GERALD M. EDELMAN

(From The Rockefeller University, New York 10021)

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Newborn mice are, in general, unable to produce humoral antibody in response to ordinary antigenic stimulation, but they acquire this capacity during the first few days or weeks after birth. It is likely that certain maturational events occurring during this time account for the onset of humoral responses. In adult mice, the production of antibodies usually requires the presence and interaction of two kinds of antigen-specific cells—bone marrow-derived lymphocytes (B cells) and thymus-derived lymphocytes (T cells). Information concerning the appearance and maturation of these cell types is necessary to an understanding of the events that control the onset of immunological competence.

In a previous study (1), we found that immunoglobulin-bearing cells (B cells),  $\theta$ -positive cells (T cells), and antigen-binding cells could all be detected in the spleens of Swiss-L mice before birth, as early as the 15th day of a 19 day gestation period. The most rapid increases in the total numbers and percentages of all these cell types occurred during the 4 days before and 2 days after birth. The onset of humoral responses to two different antigens could not be detected, however, until 1–2 wk after the most rapid emergence of T cells, B cells, and antigen-binding cells in the spleen. We concluded that the mere presence of these various lymphoid cell types, as identified by their surface molecules, was not sufficient for antibody synthesis, but that qualitative changes in the functional properties of the cells or populations of cells might be required during the first few weeks after birth.

In the present study, we have examined the capacity of spleen cells from Swiss-L mice of different ages to respond *in vitro* to antigens and to mitogens. Our results indicate that B cells from immunologically immature mice are capable of responding to antigen provided that T-cell function is substituted or enhanced by bacterial lipopolysaccharide (2–4). Spleen cells from newborn Swiss-L mice are not capable of responding to the T-cell mitogens, concanavalin A (Con A),<sup>1</sup> and phytohemagglutinin (PHA), even though 20% of the cells carry the  $\theta$ -antigen (1). Cells responsive to Con A could be detected at an earlier

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† Supported by a Postdoctoral Fellowship from the Arthritis Foundation. Present address: Department of Microbiology, The University of Chicago, Chicago, Ill. 60637.

<sup>1</sup>Abbreviations used in this paper: ATS, antiserum prepared in rabbits against mouse thymocytes; Con A, concanavalin A; LP, bacterial lipopolysaccharide; PFC, plaque-forming cells; PHA, phytohemagglutinin; SRBC, sheep red blood cells.

age than cells that can respond to both mitogens. Our results suggest that one of the last events in the development of humoral immunocompetence may be the appearance of cells with the characteristics of long-lived, recirculating, PHA-responsive and Con A-responsive T cells.

#### *Materials and Methods*

*Mice.*—Swiss-L mice from the specific pathogen-free colony maintained at The Rockefeller University were used in the present studies. These mice are randomly bred within a closed colony that was started in 1926 with nine animals (5). Breeding cages were checked every morning for new births. Age was reckoned by considering the day on which a new litter was found to be the day of birth.

*Antithymocyte Serum.*—Antithymocyte serum (ATS) was prepared in rabbits by the injection of Swiss-L mouse thymocytes according to the method of Levey and Medawar (6). The serum obtained had a cytotoxic titer of 1:300 against Swiss-L mouse thymocytes, killing more than 99% of these cells. The serum was heat inactivated at 56°C for 1 h and adsorbed three times with mouse erythrocytes at a ratio of 1 part erythrocytes to 20 parts ATS.

*Mitogens.*—Concanavalin A (Con A) was prepared from defatted jack bean meal (Schwarz/Mann Div., Becton, Dickinson and Co., Orangeburg, N. Y.) by the method of Cunningham et al. (7). The purified protein was dissolved in saline at a concentration of approximately 1 mg/ml. After Millipore filtration (Millipore Corp., Bedford, Mass.) the actual Con A concentration was determined spectrophotometrically (8) and the sterile solution was stored in aliquots at -20°C. Phytohemagglutinin (PHA) from two different commercial sources was used. Bacto-phytohemagglutinin-P (PHA-P), lot no. 552139, was obtained from Difco Laboratories (Detroit, Mich.), reconstituted with water as directed, and stored at -20°C until use. Mitogen A (PHA-A), lot no. 720908, and mitogen B (PHA-B), lot no. 720914, both prepared according to the procedure of Oh and Conard (9), were purchased from Seoney Laboratories (Houston, Tex.). These PHA preparations were obtained as lyophilized powders containing salts and were dissolved in water at 10 mg/ml, filter sterilized, and stored at -20°C in small aliquots. Bacterial lipopolysaccharides (LP), prepared from *Escherichia coli* 055:B5 (lot no. 581336) and from *Salmonella typhosa* 0901 (lot no. 589254) by the Westphal procedure (10), were purchased from Difco Laboratories. *E. coli* 055:B5 LP, lot no. 585002, was also tested but found to give complex dose-response curves, indicative of some activity that interfered with the stimulatory activity of LP. This preparation was not used in any of the studies reported here. Each LP preparation was dissolved in 0.01 M phosphate buffer, pH 8.0, at 1 mg/ml, heated to boiling for 1 h, and stored in aliquots at -20°C (11).

*Spleen Cell Suspensions for In Vitro Cultures.*—Using sterile technique, spleens were dissected from one or more littermates, depending upon the number of cells required for any given experiment. For mice younger than 1 wk of age, two or three litters born on the same day were sometimes required to obtain a sufficient number of cells. The spleen capsules were punctured many times with needles, and the cells were gently extruded from the organ and forced through a stainless steel screen into Eagle's minimal essential medium prepared with suspension salts (Microbiological Associates, Inc., Bethesda, Md.) and brought to pH 7.4 by the addition of NaOH. Cell clumps were gently disrupted by pipetting. The suspensions were transferred to sterile plastic tubes and left in an ice bath for a few minutes to allow remaining clumps to settle. After transferring the declumped suspension to another tube, the cells were pelleted by low-speed centrifugation and then resuspended in culture medium. The concentration of viable nucleated cells was determined by counting the cells that excluded trypan blue using a hemacytometer. In these preparations 75-90% of the cells were viable.

*Antigenic Stimulation In Vitro.*—For studies of the in vitro primary response to antigen,  $1.5 \times 10^7$  viable nucleated spleen cells in 1 ml of medium were cultured in 35-mm disposable

plastic dishes (Nunc, distributed by Vanguard International, Inc., Red Bank, N. J.) by the procedure of Mishell and Dutton (12) with the addition of mercaptoethanol as suggested by Click et al. (13). Components of the culture medium, formulated exactly as described by Mishell and Dutton (12), were purchased from Microbiological Associates, Inc. with the exception of the serum. The fetal bovine serum used in the culture medium (Rehatuin F. S., lot no. J75605) was obtained from Reheis Chemical Co. (Chicago, Ill.). This particular lot of serum was chosen, from among several tested from different sources, for its ability to support good primary immune responses to sheep erythrocytes (SRBC) with low background levels of antibody-secreting cells produced in the absence of added antigen. A stock solution of mercaptoethanol (0.1% vol/vol in saline) was diluted 1:6 with medium and 25- $\mu$ l aliquots were added to each culture. To some cultures, 30  $\mu$ l of 1% SRBC (Microbiological Associates, Inc.) in saline was added as the antigenic stimulus. LP, appropriately diluted with medium, was added to some cultures while maintaining the total volume at 1 ml and the cell concentration at  $1.5 \times 10^7$  viable cells per ml. Cultures were incubated at 37°C in a humidified atmosphere of 10% CO<sub>2</sub>, 7% O<sub>2</sub>, and 83% N<sub>2</sub> on a rocking platform (Bellco Glass, Inc., Vineland, N. J.) and were fed daily with a nutritional mixture as formulated by Mishell and Dutton (12). After 2–5 days of incubation, duplicate cultures were pooled. The cells were washed, resuspended in a balanced salt solution (12), and then assayed for the presence of cells secreting antibodies to SRBC using the Jerne plaque technique (14).

*Mitogenic Stimulation In Vitro.*—For mitogenic assays, 0.2-ml aliquots of spleen cells at  $1.5 \times 10^7$  viable nucleated cells per ml were mixed in duplicate with 0.1-ml aliquots of mitogen, appropriately diluted from stock solutions with medium. The mixtures were cultured in loosely capped, disposable plastic tubes (12  $\times$  75 mm [Falcon Plastics, Div. of BioQuest, Oxnard, Calif.]) in a humidified atmosphere of 10% CO<sub>2</sub>, 7% O<sub>2</sub>, and 83% N<sub>2</sub>. The culture medium was formulated as described by Mishell and Dutton (12) and the cultures were fed daily with 0.03 ml nutritional mixture according to their procedure (12). After 46 h of incubation, 0.1 ml of culture medium containing 1  $\mu$ Ci of [<sup>3</sup>H]thymidine, sp act of 1.9 Ci/mmol (Schwarz/Mann), was added to each tube. After an additional 22 h of incubation, the cells were collected by filtration on glass fiber filters (Whatman GF/A) and washed sequentially with 5% trichloroacetic acid and methanol. The filters were dried and counted by liquid scintillation spectroscopy.

## RESULTS

*Age Dependence of In Vitro Primary Response to SRBC.*—Spleen cells from Swiss-L mice of different ages were cultured in the presence and absence of SRBC in order to determine their in vitro response to an antigenic stimulus. Mercaptoethanol was added to all cultures because this procedure has been shown to restore the plaque-forming capacity of adult mouse spleen cells from which the adherent cells or macrophages had been removed (15). In this way, attention could be focused on the maturation of lymphocyte function without the added complication of controlling for changes in macrophage numbers with age.

The numbers of plaque-forming cells (PFC) induced in response to SRBC increased with increasing age of the spleen donors (Fig. 1). This observation parallels the results reported earlier for the in vivo responses of these mice to SRBC (1). That is, 1-wk old mice gave little or no detectable response, but the response increased thereafter to reach adult levels by 6–8 wk of age. It should be pointed out, however, that the parallel between the in vitro and in vivo

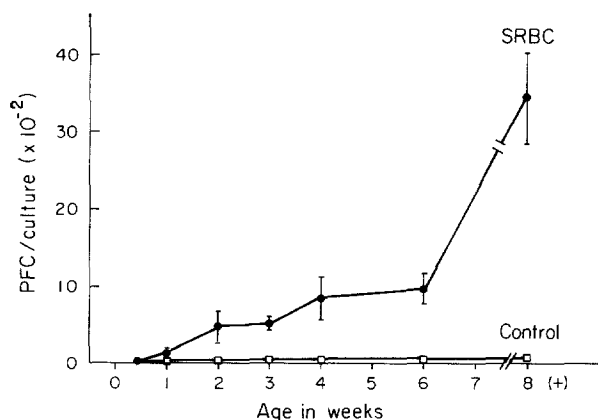


FIG. 1. Numbers of PFC produced by spleen cells *in vitro*, in the presence and absence of SRBC as an antigenic stimulus, as a function of the age of the spleen donors. Cultures were assayed on the 5th day of incubation. Each point represents the mean calculated from values obtained in four to nine independent experiments. The vertical bars represent the standard error of the mean.

systems is probably fortuitous inasmuch as different factors may limit the immune response in the two experimental systems. For example, insufficient numbers of macrophages would probably not affect the *in vitro* response under the conditions used but the *in vivo* response might be severely impaired. Also, allogeneic effects probably somewhat enhance the *in vitro* immune responses of spleen cells from the Swiss-L mice. We have observed that cells prepared from a single adult mouse generally give lower responses to SRBC than a pool of cells from littermates. Because of the numbers of cells usually required, pools of cells were used in all of the experiments reported here.

The most important point to be gained from the *in vitro* data presented in Fig. 1, and from the *in vivo* data previously reported (1), is that the primary response to antigen in Swiss-L mice is very low at 3 wk of age and practically undetectable at 1 wk of age despite the fact that the percentages of spleen cells identified by their surface markers as B lymphocytes, as T lymphocytes, or as SRBC-binding cells are relatively high at these ages (1). The possibility, therefore, emerged that either the B cells or the T cells, or both, are functionally immature.

*Effect of LP on the In Vitro Response to Antigen.*—The immunologic capacity of the B cells in immature mice was investigated by asking whether these cells could be made to respond to antigen under any conditions, i.e., are the B cells fully functional but unable to undergo maturation to antibody-secreting cells in response to antigen because T-cell helper function is absent? This particular situation exists in the lymphoid organs of nude mice, which are congenitally athymic. Several workers (2, 3) have reported that spleen cells from nude mice

can be made to respond to antigen in the presence of lipopolysaccharides (LP) that have been extracted from the cell walls of gram-negative bacteria (10). The LP either provides a stimulus to B cells that T cells usually provide (11, 16) or possibly induces the differentiation of T cells (4). Whatever its mode of action, it was of interest to determine whether LP could enhance the *in vitro* primary immune responses of spleen cells from immunologically immature Swiss-L mice. The results shown in Fig. 2 demonstrate that LP preparations from both *S. typhosa* and *E. coli* increased the number of PFC that could be induced by SRBC in spleen cells from mice ranging in age from 1 wk to 8 wk or

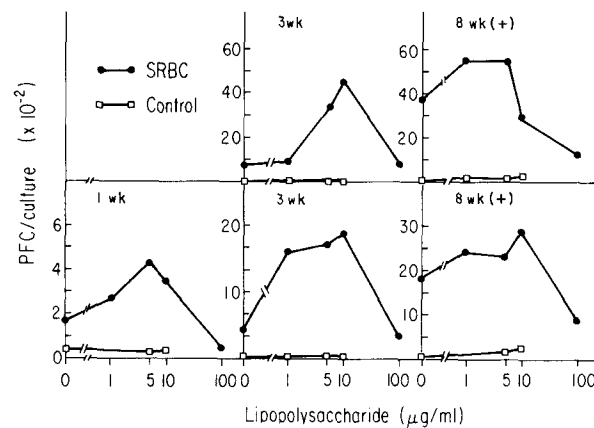


FIG. 2. Effect of LP on the induction of PFC *in vitro* in the presence and absence of SRBC as an antigenic stimulus. LP, extracted from either *S. typhosa* or *E. coli*, was present from the beginning in cultures prepared from the spleens of Swiss-L mice at 1, 3, or 8 wk (+) of age. In the particular experiments illustrated here, *S. typhosa* LP was used in the cultures from 1-wk old mice, *E. coli* LP was used in those from 3-wk old mice (two independent experiments top and bottom panels), and each was used separately in the cultures from 8-wk old mice (top panel, *E. coli*; bottom panel, *S. typhosa*). The cultures were assayed on the 5th day of incubation.

older. The optimal concentration for this effect, assayed on the 5th day of culture, was 5–10  $\mu\text{g}/\text{ml}$ , regardless of the source of LP. No significant differences in the effects of *S. typhosa* LP and *E. coli* LP were detected.

Table I presents the data accumulated from a number of experiments done to test the effect of LP on the PFC response to SRBC. The results are shown only for the concentration of LP that gave the highest response in each experiment. As measured on the 5th day of culture, LP added in the absence of SRBC induced slightly higher than background levels of PFC. The LP-induced enhancement of the specific response to SRBC was smallest for mice 8 wk of age and older, intermediate for 3-wk old mice, and largest for 1-wk old mice. It appears that the lower the response of the spleen cells to SRBC alone, the greater the enhancement in the presence of LP. The numbers of PFC that could be detected

on day 5 in cultures containing both SRBC and LP were within the same range of values for 3-wk old and adult mice but were somewhat below this range for 1-wk old animals. In this connection, it may be significant that B cells constitute 29% of the nucleated cells in the spleens of 1-wk old mice, compared with 53% for adults (1).

Because the production of antibody-secreting cells in response to antigen involves both the proliferation and differentiation of B cells, it was of interest to examine the appearance of PFC with time in cultures maintained in the presence and absence of LP. The results presented in Fig. 3 show that the kinetics of appearance of PFC in the presence of SRBC and LP were similar for 3-wk old

TABLE I  
*The Effect of Lipopolysaccharide on the PFC Response*

Age	LP		PFC/culture				SRBC + LP/SRBC
	Species	Conc.	No additions	LP	SRBC	SRBC + LP	
<i>wk</i>		$\mu\text{g/ml}$					
1	<i>E. coli</i>	5	0	13	8	170	21
	" "	10	50	65	40	840	21
	" "	10	5	n.t.*	130	1,170	9
3	<i>E. coli</i>	10	10	38	710	4,480	6
	" "	10	25	20	435	1,880	4
	" "	10	18	65	570	2,260	4
6	<i>S. typhosa</i>	10	58	195	1,520	2,830	1.9
8 (+)	<i>S. typhosa</i>	10	30	240	1,810	2,870	1.6
	<i>E. coli</i>	10	35	73	1,060	1,670	1.6
	" "	5	33	160	3,710	5,460	1.5
	" "	10	45	n.t.	4,090	4,970	1.2

\* Not tested.

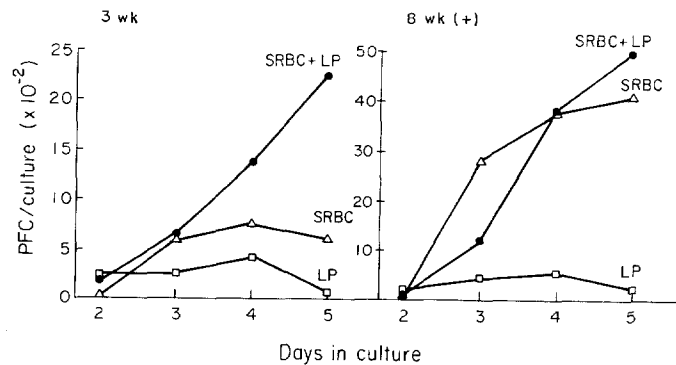


FIG. 3. Kinetics of PFC induction in vitro in the presence of SRBC plus LP, SRBC alone, and LP alone. The results presented here are representative of several experiments done with spleen cells from mice at 3 and 8 wk (+) of age, using the LP from *E. coli* 055:B5 at 10  $\mu\text{g/ml}$ .

and adult mice. In the presence of SRBC alone, however, the cultures prepared from 3-wk old animals could not support the proliferation and/or differentiation of B cells. We conclude that, in the presence of LP, B cells from immunologically immature mice and from adult mice are capable of responding to antigen in a similar fashion, in terms of both the kinetics and the magnitude of the response. These results suggest the possibility that the humoral immunological defects of young Swiss-L mice are due, not to the inability of B cells to function, but to the immaturity of helper T cells.

*Response of Spleen Cells to T-Cell Mitogens.*—Subpopulations of T cells in adult mice have been characterized by a number of parameters (17, 18), including their ability to be stimulated by the mitogens, Con A, and PHA (19). It has been reported that T cells with the properties of long-lived recirculating lymphocytes respond both to Con A and PHA, while other T cells that reside primarily in the spleen probably respond only to Con A (19). Experiments were carried out to determine whether cells that could respond to these two mitogens were present in the spleens of immunologically immature Swiss-L mice. Because of the possibility that optimal concentrations for stimulation would vary with the age of the spleen donors, Con A and PHA were tested at several different concentrations for their ability to stimulate the incorporation of [<sup>3</sup>H]thymidine into DNA. Gunther et al.<sup>2</sup> have shown that the amount of [<sup>3</sup>H]thymidine incorporated is proportional to the number of cells responding.

The results shown in Fig. 4 demonstrate that maximal mitogenic stimulation was achieved with 2–5  $\mu$ g of Con A per ml for spleen cells from mice up to 4 wk of age and for the majority of cells from older animals. The biphasic dose-response curve found for cells from mice 8 wk of age and older was highly reproducible; it was observed in each of the five independent experiments from which the data were pooled to compute the means shown in Fig. 4. This biphasic curve suggested the presence of a new cell population that responded optimally to 1  $\mu$ g of Con A per ml. Smaller numbers of these cells were probably also present in 3- and 4-wk old mice, judging from the asymmetry of the dose-response curves. As shown in Figs. 4 and 6, 1-day old mice had very few Con A-responsive cells in their spleens, but the numbers of these cells increased sharply with age, reaching a maximum at about 3 wk.

PHA-responsive cells appeared in the spleen at a later age than Con A-responsive cells, as shown in Figs. 5 and 6. The concentration of PHA required for optimal stimulation did not vary with the age of the spleen donors. Both PHA-A and PHA-B, prepared by Seeney Laboratories according to the procedure of Oh and Conard (9), were optimally stimulatory at 100  $\mu$ g of lyophilized powder (containing material other than protein) per ml. PHA-P from Difco Laboratories was optimally active at 0.15–0.3  $\mu$ l per ml. All three PHA preparations gave the same levels of stimulation with a single suspension of spleen cells. It should be pointed out that any allogeneic differences between spleen cells from

<sup>2</sup> Gunther, G. R., J. L. Wang, and G. M. Edelman. Manuscript in preparation.

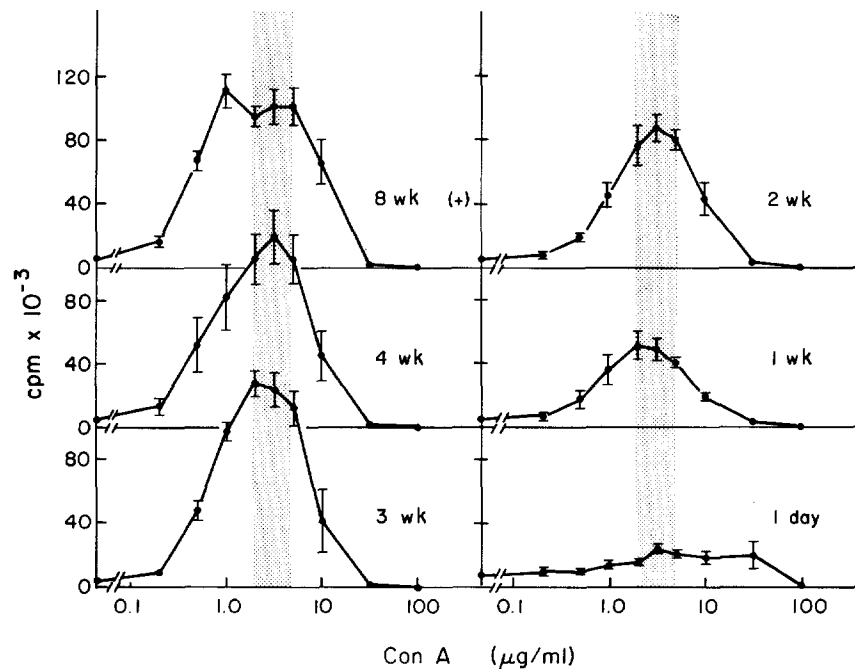


FIG. 4. Mitogenic stimulation of spleen cells from mice of different ages as a function of Con A concentration. The amount of [ $^3\text{H}$ ]thymidine incorporated into DNA during the period from 46 to 68 h of culture was quantitated as described in Materials and Methods. Each point represents the mean of three to five independent determinations and the vertical bars represent the standard error of the mean. The shaded strips span the range of Con A concentrations (2–5  $\mu\text{g}/\text{ml}$ ) that was maximally stimulatory for spleen cells from animals 4 wk of age and younger. Each dose-response curve obtained in five independent experiments with spleen cells from mice 8 wk of age or older resembled the biphasic curve drawn from the computed means and shown here.

littermates had no observable effects on the mitogenic stimulation of these cells by Con A or PHA, inasmuch as similar results were obtained whether cells from a single mouse or cells pooled from littermates were tested.

*Effects of ATS on Cells that Respond to Con A and PHA.*—The cells that were stimulated by PHA and those that responded optimally to 1  $\mu\text{g}$  of Con A per ml both appeared in the spleen at about 3 wk of age (Figs. 4 and 6). This observation suggested that the two activities might be properties of the same cell population. To test this hypothesis, adult mice were injected intravenously with 0.5 ml of ATS, a procedure that has been shown to deplete the spleen of PHA-responsive cells and, to a lesser extent, of Con A-responsive cells (19). 3 days after the administration of ATS, spleen cells were prepared from treated and control mice and were tested for their responses to Con A and to PHA. The results shown in Fig. 7 confirm the finding that ATS treatment depletes the



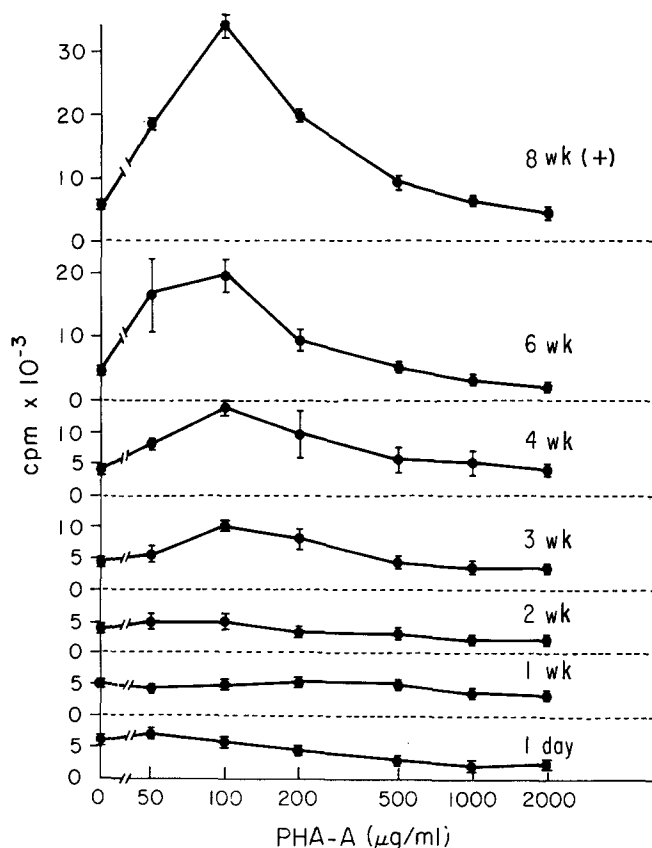


FIG. 5. Mitogenic stimulation of spleen cells from mice of different ages as a function of PHA-A concentration, expressed as the weight of commercial lyophilized powder (protein and salts) per unit volume of culture medium. The amount of [ $^3\text{H}$ ]thymidine incorporated into DNA during the period from 46 to 68 h of culture was quantitated as described in Materials and Methods. Each point represents the mean of three independent determinations and the vertical bars represent the standard error of the mean.

spleen of PHA-responsive cells. In addition, we found that the cells responsive to  $1 \mu\text{g}$  of Con A per ml were also depleted, while the cells responsive to higher doses of Con A were relatively unaffected. The data suggest that sensitivity to mitogenic stimulation by low doses of Con A ( $1 \mu\text{g}/\text{ml}$ ) is another property that distinguishes the PHA-responsive, ATS-sensitive T cells from other T cells.

A comparison of the magnitude of *in vivo* humoral responses to SRBC as a function of age (1) with the time of appearance of PHA-responsive cells (Fig. 6) suggests the hypothesis that the limiting step in the onset of humoral immune responses may be the appearance of lymphocytes with characteristics of the ATS-sensitive, or recirculating (20), T cells. This positive correlation between

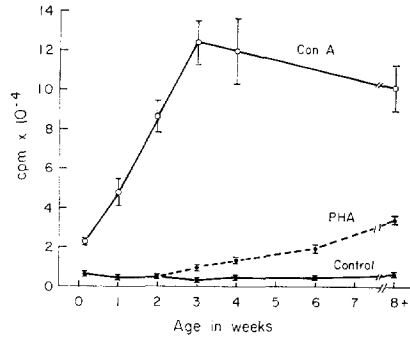


FIG. 6. Mitogenic stimulation of spleen cells from mice of different ages by Con A at 3  $\mu\text{g}/\text{ml}$  and by PHA-A at 100  $\mu\text{g}/\text{ml}$ . The bulk of the cells at each age responded best to these concentrations of Con A and PHA (Figs. 4 and 5). The data presented were taken from Figs. 4 and 5.

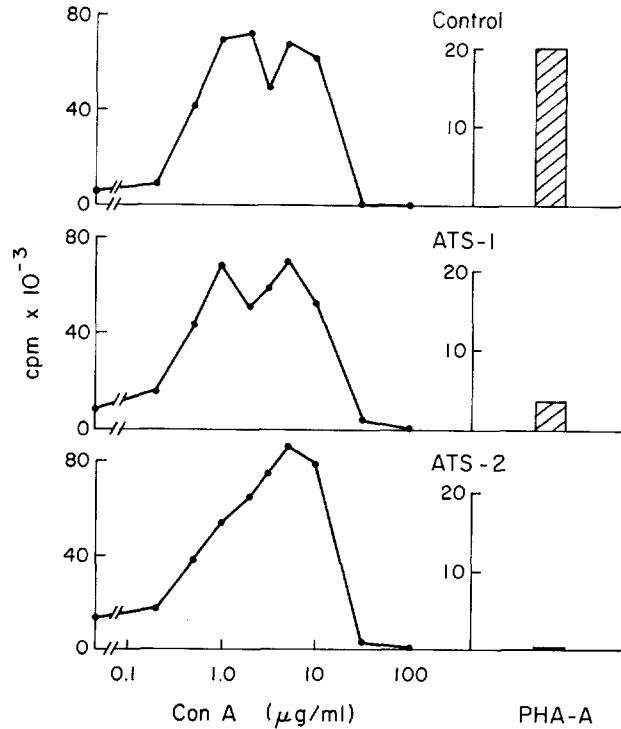


FIG. 7. Effect of the in vivo administration of ATS on the mitogenic stimulation of spleen cells by Con A and PHA-A. 3 days after 10 wk old mice had been injected intravenously with ATS (0.5 ml), spleen cells were prepared from treated and control mice and tested for their responses to Con A at several concentrations and to PHA-A at 100  $\mu\text{g}/\text{ml}$ . Conditions for measuring the incorporation of [<sup>3</sup>H]thymidine into DNA were the same as described in the legends to Figs. 4 and 5.

the capacity to produce antibody in response to antigen and the appearance during development of recirculating T cells that respond both to PHA and to Con A at 1  $\mu\text{g}/\text{ml}$  is strengthened by the finding that the ATS treatment of adult mice depresses the primary humoral immune response (21-23) as well as depletes the spleen of these cells.

#### DISCUSSION

Swiss-L mice, at 1 wk of age, are unable to produce detectable amounts of antibody in response to ordinary antigenic stimulation, either *in vivo* (1) or *in vitro*. Low levels of response can be measured at 2 wk of age, but full maturation of the humoral immune response appears to require another 4-6 wk. The development of this capacity to synthesize antibodies after an antigenic stimulus lags behind the appearance of immunoglobulin-bearing B cells and  $\theta$ -positive T cells in the spleens of these mice (1). The results presented here demonstrate that the B cells of immunologically immature mice function similarly to adult B cells in at least one respect: in the presence of bacterial LP, both could be induced by antigen to produce PFC with similar kinetics and levels of response. The  $\theta$ -positive cells in the spleens of young Swiss-L mice are demonstrably different, however, from adult splenic T cells in their responses to two different mitogens. The numbers of cells in the spleen that could respond to Con A increased sharply from birth to 3 wk of age but the dose-response profiles at 3 wk differed from those found in adults. On the other hand, cells that could respond to PHA were not detected until 3 wk of age and did not reach adult levels until 8 wk. There is a positive correlation between the appearance of the PHA-responsive T cells and the ability of the mice to respond to antigen by the production of humoral antibody.

The ability of LP to stimulate *in vitro* PFC responses to antigens in the absence of helper T cells was demonstrated using spleen cells from nude mice (2, 3). These mice have functional B cells but few or no T cells, and it has been suggested that the LP acts directly on the B cells to provide a stimulatory signal usually provided by activated T cells (11, 16). This hypothesis seems to be supported by the finding that LP is apparently a B-cell mitogen inasmuch as it can stimulate DNA synthesis in spleen cells that are essentially devoid of T cells (24, 25). Some recent findings suggest, however, that other kinds of cells may be stimulated by LP, either directly or indirectly. Effenbein et al. (26) have demonstrated that, when spleen cells from BALB/c mice are incubated with LP, only 26% of the cells that are stimulated to make new DNA can be identified as B cells by their complement receptors. Although it seems possible that some of the unidentified stimulated cells could be B cells that had lost their complement receptors, these cells have not yet been characterized. Furthermore, it remains to be determined whether similar results would be obtained using spleen cells from nude mice. A more startling result relating to the activity of LP comes from the work of Sheid et al. (4). They have found that the incuba-

tion of spleen cells from nude mice with LP, or with a number of other agents, results in the appearance of  $\theta$ - and TL antigens on cells that were previously negative for these antigens. Their data suggest that LP can perhaps induce the differentiation of T-cell precursors present in nude mouse spleens.

The mechanism by which LP induces PFC responses to antigen in the apparent absence of helper T-cell function has relevance to an interpretation of the data presented here. If LP does not act directly on B cells but by inducing the functions of helper cells, then our results would strongly suggest that the B cells of young immunologically incompetent mice do not differ demonstrably in function from the B cells of adults. If, however, LP acts directly on B cells, then the possibility exists that LP could induce an antigen-specific response in immature B cells that would ordinarily not be able to respond to antigen, even under the influence of helper T cells. This uncertainty can be simply resolved by determining whether purified T cells from adult mice can enhance the PFC response to antigen of spleen cells from young mice. These experiments will have to be carried out in inbred mice in order to avoid the complication of stimulatory effects due to allogeneic differences.

There are at least two subpopulations of T cells in adult mice (17-19). The selective effects of ATS treatment *in vivo* have been useful in characterizing these two kinds of cells. The cells most sensitive to the action of intravenously injected ATS are thymus-derived, long-lived, recirculating lymphocytes (20) that respond to mitogenic stimulation by both PHA and Con A (19) and are important for both cell-mediated immunity (6, 27) and the primary humoral response (21-23, 28). The thymus-derived cells that are relatively insensitive to ATS reside primarily in the spleen (17-19) and are probably responsive only to Con A (19). The results presented here indicate that these two cell populations differ in yet another respect. Careful studies of the mitogenic stimulation of adult spleen cells as a function of Con A concentration suggested the existence of two cell populations that responded optimally to different concentrations of Con A. This suggestion was supported by the finding that ATS treatment depleted one cell population but not the other. The ability to respond better to the lower dose of Con A (1  $\mu\text{g}/\text{ml}$ ) than to the higher dose (3  $\mu\text{g}/\text{ml}$ ) appears to be a property of the recirculating PHA-responsive cells since cells that can be stimulated by PHA and by 1  $\mu\text{g}$  of Con A per ml arise together during the development of Swiss-L mice and are depleted together by ATS treatment.

At birth, 20% of the spleen cells in Swiss-L mice are  $\theta$ -positive and therefore of thymic origin as compared with 27% in adult mice (1). These  $\theta$ -positive spleen cells of neonatal and young mice differ from those of adult mice in several respects. Although some of the  $\theta$ -positive cells from newborns may be capable of binding to antigen, as has been demonstrated for adult T cells (29-32), most of them probably cannot bind antigen with avidities sufficient to allow detection in the usual assays (1). Furthermore, as shown by the results presented here, the  $\theta$ -positive spleen cells from young mice are not sensitive to stimula-

tion by Con A and PHA to the same extent as adult cells. All these observations suggest the possibility that the  $\theta$ -positive cells in young mice must undergo further maturational events before they can perform their proper immunologic functions. This hypothesis is consistent with the late development of humoral immune responses in spite of the early presence of  $\theta$ -positive cells and of B cells that can respond to antigen under conditions such that LP can supply or enhance helper function.

Although the relationship between sensitivity to specific mitogens and the maturation of immunologic functions in T cells is not yet clearly defined, responsiveness to mitogens has proved to be a useful tool for the characterization of T-cell subpopulations in the adult (19). Cells that can be stimulated by Con A at 3  $\mu\text{g}/\text{ml}$  appear at an earlier age in the spleens of Swiss-L mice than those ATS-sensitive cells that respond to PHA and to Con A at 1  $\mu\text{g}/\text{ml}$ . The maturation of humoral immune responses in these mice appears to be correlated with the appearance of the latter cells. That lymphocytes with the characteristics of the ATS-sensitive cells are important for the primary humoral response has been demonstrated by the finding that ATS treatment depresses this response (21-23). It therefore seems a likely hypothesis that the limiting step in the development of full humoral immune responses in Swiss-L mice is the appearance, or generation from other T cells, of cells with the properties of recirculating, PHA-responsive and Con A-responsive T lymphocytes.

#### SUMMARY

In spite of the prenatal appearance of immunoglobulin-bearing lymphocytes and  $\theta$ -positive lymphocytes in the spleens of Swiss-L mice, these mice are not able to produce detectable levels of humoral antibodies in response to antigen until after 1 wk of age. Adult levels of response are not achieved until 4-8 wk of age. In the presence of bacterial lipopolysaccharides, which can substitute for or enhance T-cell function, the B cells from young Swiss-L mice were found to be indistinguishable in function from adult B cells, both with respect to the numbers of plaque-forming cells (PFC) produced in vitro in response to antigen and with respect to the kinetics of PFC induction. The spleen cells from young Swiss-L mice are significantly less sensitive than adult spleen cells, however, to stimulation by the T cell mitogens, concanavalin A (Con A) and phytohemagglutinin (PHA). Very few Con A-responsive cells could be detected at birth but the numbers increased sharply with age until 3 wk after birth. On the other hand, PHA-responsive cells could not be detected in the spleen until about 3 wk of age. The latter cells were found to respond also to Con A, but at a lower dose (1  $\mu\text{g}/\text{ml}$ ) than that required for the bulk of the Con A-responsive cells (3  $\mu\text{g}/\text{ml}$ ). The cells that respond both to PHA and to Con A appear in the spleen at about the time that Swiss-L mice acquire the ability to produce humoral antibodies, and these cells can be depleted from the spleen by the in vivo administration of antithymocyte serum. The development of humoral immune

responses in these mice therefore appears to be correlated with the appearance of recirculating T lymphocytes that are responsive both to PHA and to Con A.

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