

## INDUCTION OF IgG BY LIPID A IN THE NEWBORN MOUSE\*

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Lipopolysaccharides (LPS)<sup>1</sup> from gram-negative bacteria are powerful mitogens for B cells in vitro (1). In contrast to antigens LPS stimulates a broad spectrum of B cells regardless of their specificity for antigen (1,2). The mitogenic part of the LPS molecule has been found to be the lipid A component (3).

Lipid A stimulation in vitro of cells from nonimmunized adult mice resulted in induction of IgM synthesis only (2). We wish to report results which show that IgG synthesis may be induced, provided lipid A is given to neonatal mice in vivo. In agreement with results obtained in vitro (1) we could not demonstrate an in vivo IgG stimulation in adult mice. In order to measure newly synthesized IgG in the newborn, a sensitive hemolytic assay was developed, which permits the determination of IgG of paternal allotype in concentrations of a few micrograms per milliliter.

### Materials and Methods

*Animals.*—Offspring from reciprocal matings between BALB/c/AnNIcr (allotype-a) and C57BL/6NIcr (allotype-b), which were heterozygous for the IgG allotypic marker, were used.

*Lipid A and Salmonella.*—Lipid A was obtained by acid hydrolysis (4) of phenol-water-extracted LPS (5). 1 mg lipid A was dissolved in 1 ml distilled water by addition of 0.5  $\mu$ l triethylamine (6). *Salmonella anatum* was cultivated in nutrient broth, inactivated by 1% phenol, washed in distilled water, and lyophilized. For injection, preparations were diluted in phosphate-buffered saline (PBS).

*Treatment of Mice.*—On various days after birth some mice of each litter were injected intraperitoneally with 0.03 mg of lyophilized *Salmonella anatum* or with 17  $\mu$ g lipid A/gram body weight. The remaining two to three mice of each litter served as controls. They were

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<sup>1</sup> Abbreviations used in this paper: An, natural unit of antibody; anti-a, anti-b, antiallotype antisera; BSA, bovine serum albumin; DNP-KLH, dinitrophenyl-keyhole limpet hemocyanin; EDTA/PBS, 0.012 M ethylene-diamine-tetraacetate in PBS; LPS, lipopolysaccharide; M + H, Mayer's barbital buffer supplemented with 0.1% human serum albumin; NKM/PBS, solution of 0.13 M NaCl, 0.005 M KCl, and 0.07 M MgCl<sub>2</sub> in PBS 1:1; PBS, phosphate-buffered saline; SRBC, sheep red blood cells.

injected either with PBS alone, or with 2  $\mu$ g bovine serum albumin (BSA), or were left untreated.

Every two to three days after injection some mice were exsanguinated. From the 12th to 15th day after birth sequential bleeding was performed by retroorbital puncture.

*Antisera.*—Antisheep red blood cell antiserum of allotype-a was prepared in BALB/c mice, or in C57BL mice for antibodies of allotype-b, by three weekly injections of  $10^8$  sheep red blood cells (SRBC). Anti allotype-a antiserum, prepared as described (7, 8), reacts with IgG1<sup>a</sup> (MOPC 31) and IgG2a<sup>a</sup> (MOPC 173) myelomas from BALB/c mice and not with IgG2b<sup>a</sup> (MOPC 195) or IgA (MOPC S117). Anti allotype-b antiserum, prepared in BALB/c mice reacts with IgG from C57BL mice, probably with IgG2a<sup>b</sup>, as no precipitation with IgG1<sup>b</sup> (MOPC 300) or IgG2b<sup>b</sup> (MOPC 352) was detectable. For detailed discussion see reference 9. The anti allotype antisera were absorbed with SRBC. The same batches of antisera were used throughout this study.

*The Quantitative IgG-Test.*—The test is based on a quantitative indirect hemolysis assay (10). Mouse anti-SRBC antibodies of the IgG class, bound to SRBC (“sensitization”), are not efficient in complement fixation (i.e., do not cause direct lysis). Complement-dependent hemolysis may be obtained by complexing the sensitized SRBC with antibodies directed against the allotypic determinants of the bound anti-SRBC antibodies; these complexes fix complement leading to hemolysis (indirect lysis; “facilitation”). The IgG assay as modified for our purposes makes use of an inhibition reaction: IgG molecules carrying the same allotype as the sensitizing anti-SRBC will compete for the facilitating anti allotype serum, and consequently a lower degree of hemolysis will be obtained. Standardization with varying concentrations of a normal serum (standard) will give an inhibition curve. Any serum with unknown quantities of IgG of a given allotype, when incubated with the corresponding anti allotype serum used for facilitation, will thus inhibit hemolysis. By comparison with the standard curve the inhibitory power of the test serum can be calculated. Inhibition activity was taken as a measure of relative concentrations of IgG bearing the allotype in question.

*Experimental Procedure.*—

*Sensitization:* SRBC, washed three times in a solution of 0.13 M NaCl, 0.005 M KCl, and 0.07 M MgCl<sub>2</sub> in PBS1:1 (NKM/PBS), were diluted with Mayer's barbital buffer supplemented with 0.1% human serum albumin (M + H) such that an aliquot of 50  $\mu$ l when lysed in 700  $\mu$ l of 0.1% Na<sub>2</sub>CO<sub>3</sub> gave an optical density of 0.800 at 541 nm. Anti-SRBC (of either allotype-a or allotype-b) were treated with 0.2 M 2-mercapto-ethanol for 30 min at 37°C to eliminate IgM antibodies that might cause direct lysis. The diluted standard sera were added dropwise with stirring to equal volumes of washed cells, and the reaction was allowed to continue for 1 h at room temperature. The sensitized SRBC were then washed three times in NKM/PBS, and resuspended in M + H to  $5 \times 10^8$  SRBC/ml (OD = 0.700) after  $\times 7.5$  dilution and lysis in 0.1% Na<sub>2</sub>CO<sub>3</sub>.

*Facilitation:* Either 250  $\mu$ l diluted anti allotype-a antiserum, or anti allotype-b antiserum, was added to an equal volume of sensitized SRBC. After 90 min incubation at room temperature, the reaction was stopped by diluting an aliquot of 200  $\mu$ l into 1 ml ice-cold M + H. The SRBC were separated from free antibodies by centrifugation at 12,000 rpm for 1 min in an Eppendorf Minifuge (Eppendorf, Inc., Hamburg) and resuspended in 100  $\mu$ l cold M + H.

For the determination of IgG of allotype-b, the following additional step was used. 50  $\mu$ l anti allotype-a antiserum diluted 1/100 was added to the complex of anti-SRBC and anti allotype-b antibodies. It binds to the allotype-a determinants on the anti allotype-b antibodies, and these complexes fix complement effectively. This “superfacilitation” was necessary to obtain a “single” hit curve with anti-b (see section below on calculation). In the allotype-a test the samples get 50  $\mu$ l M + H instead of superfacilitating serum, to give the same volumes as in the allotype-b test.

*Inhibition of facilitation:* The anti allotype antiserum (at a concentration of 10  $\mu$ l/ml) was preincubated with the same volume of a standard serum (pool of normal adult BALB/c or

C57BL serum, respectively) in various concentrations. As a control, anti-allotype antiserum was mixed with the same volume of M + H (inhibition control). After 1 h incubation at room temperature, the samples were diluted and used for the facilitation reaction.

*Hemolysis:* After addition of 100  $\mu$ l of diluted guinea pig complement (7HD<sub>50</sub>) (11), each sample was incubated for 50 min at 37°C and hemolysis was then stopped by the addition of 500  $\mu$ l cold 0.012 M EDTA/PBS. After centrifugation, the OD of the supernate was measured at 541 nm in a Zeiss Ultraphot Spectralphotometer (Carl Zeiss, Inc., Oberkochen, Germany).

*Calculation.*—The quantitative calculation of the assay is based on the determination of antibody concentration in natural units, An (10) of the reagents. For a “single hit” reaction, the degree of hemolysis  $I_{1ys}/I_o$  is given by

$$\frac{I_{1ys}}{I_o} = 1 - e^{-An}, \quad (1)$$

where  $I_o$  = OD of supernate when the total number of SRBC is lysed,  $I_{1ys}$  = OD of supernate after antibody-complement lysis.

If  $An = 1$  (i.e., if there is one effective antibody molecule per red cell) we calculate from Eq. 1 that

$$\frac{I_{1ys}}{I_o} = 1 - e^{-1} = 0.63.$$

that is, the degree of hemolysis is 63%, when the antibody concentration is 1 An. For an arbitrary degree of lysis Eq. 1 becomes

$$An = -\ln \frac{(I_o - I_{1ys})}{I_o}, \quad (2)$$

As mentioned above (see section on facilitation) lysis by the anti-b antiserum did not obey these equations unless superfacilitation with anti-a antibodies was used. Hence, all anti-b allotype titrations reported here were performed with superfacilitation. All reagents except the one being titrated were in excess. Table I shows the amount of various sera which give a concentration of 1 An in the standard system ( $5 \times 10^8$  SRBC/ml). The inhibition of facilitating antibodies by IgG in the standard serum results in  $An_{test}$  values which are reduced compared to the  $An_o$  of the noninhibited control. The degree of inhibition is defined to be

$$y = 1 - \frac{An_{test}}{An_o}. \quad (3)$$

Log  $y/1 - y$  is plotted against the log concentration of an inhibitory serum according to von Krogh (11) in order to obtain a “standard inhibition curve” (Fig. 1). The relative IgG con-

TABLE I  
Amount of the Different Sera Giving 1 An (63% Lysis), and Their Concentrations Used in the Assay (Microliters of Anti-serum per Milliliter of the Reaction Mixture)

Antiserum	$\mu$ l for 1 An	$\mu$ l in the assay
Anti-SRBC of allotype-a	0.046	0.25
Anti-SRBC of allotype-b	0.063	0.25
Anti-a	0.572	0.6
Anti-b	0.090	0.1

centration of an unknown serum is obtained by interpolation from the standard inhibition curve.

### RESULTS

#### *Effect of Lipid A on the IgG2a<sup>b</sup> Levels in Newborn Mice.—*

*Normal mice:* The IgG2a immunoglobulin class of the paternal allotype-b was first detectable, with this method, in untreated mice at the age of 12 days (Figs. 2 and 3), the relative concentration being about  $5 \times 10^{-4}$  of adult serum. Within 16 days the levels increase about 100-fold and reach adult levels at an age of 5 wk.

*Lipid-A treated mice:* Newborn mice were treated once with 17  $\mu$ g lipid A (for dose dependence see Table II) at different times after birth, either on day 0, 2, 4, or 7. In all groups lipid A treatment resulted in an earlier appearance of

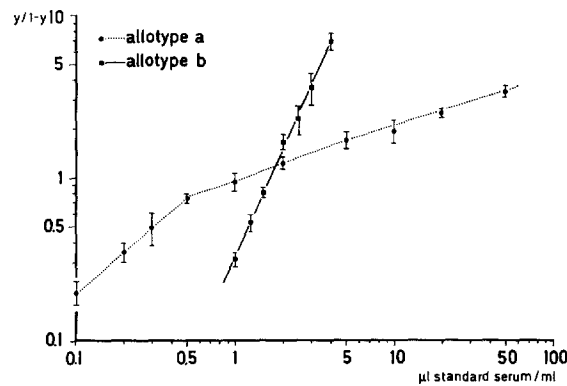


FIG. 1. Standard inhibition curve. The ordinate is  $y/1 - y$  where  $y$  is the degree of inhibition, the abscissa is the amount (microliter per milliliter buffer) of standard adult serum used for inhibition. Bars represent the standard error. (a) Allotype-a assay for IgG1<sup>a</sup> + IgG2a<sup>a</sup>; (b) allotype-b assay for IgG2a<sup>b</sup>.

TABLE II

#### *Dose Dependence of IgG2a<sup>b</sup> Stimulation by Lipid A*

Lipid A	Factor of stimulation*
<i><math>\mu</math>g/g body weight</i>	
0.017	1.0-1.3
0.17	1.7-4.5
1.7	2.8-3.3
17.0	5.4-17.5

\* Three to five mice per different dose were injected on day 7 after birth, their IgG2a levels measured on day 15 and compared to the levels in untreated mice. Higher doses resulted in higher factors of stimulation, but difficulties were encountered in injecting the higher volumes needed.

detectable IgG2a (Fig. 2); paternal IgG was detectable on day 7, as compared to day 12 in the controls. Obviously the day 7 injected mice show their first detectable IgG on day 11.

The magnitude of increase in IgG2a levels seems to depend on the age at injection, the highest levels being obtained in mice injected on day 7 and the lowest in mice injected on day 0. Thus, lipid A treatment results in an enhancement of IgG2a levels which are 3- to 30-fold higher (day 0-7, respectively) than in unstimulated mice compared at 3 wk of age.

*IgG2a<sup>b</sup> Levels in Adult Mice After Lipid-A Treatment.*—Adult mice (8 wk)

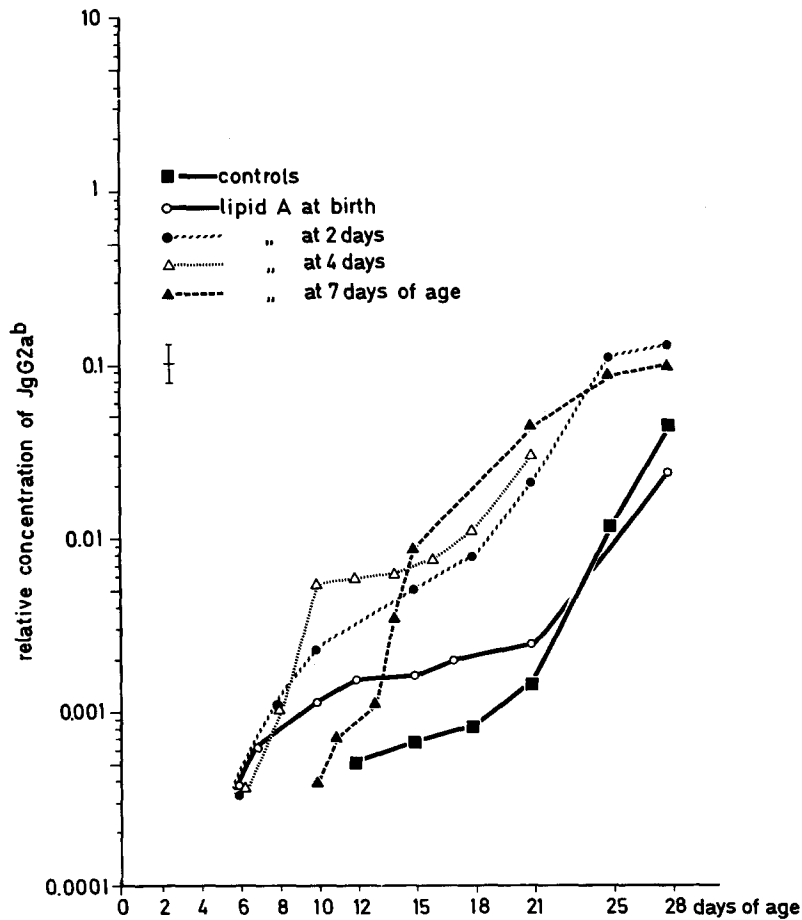


FIG. 2. Relative concentrations of IgG2a<sup>b</sup> in sera from (BALB/c × C57BL/6)F<sub>1</sub> mice treated neonatally with 17 µg lipid A/g body weight. Each point is the mean of values from six mice. The standard error of the mean for a group of six mice is 0.095 and is shown by a bar on the graph.

were injected intraperitoneally with 17  $\mu$ g lipid A/g body weight. Their serum IgG2a<sup>b</sup> levels were tested before injection and 6 days thereafter. In contrast to the results obtained in young mice no change in IgG2a levels could be observed (Table III).

*IgG2a<sup>b</sup> Levels After Salmonella Injection into Newborn Mice.*—Injection of 0.03 mg lyophilized *Salmonella anatum* also enhances the IgG2a<sup>b</sup> levels (Fig. 3). Unlike lipid A-injected mice, where the effect is sustained over many weeks, the *Salmonella*-treated mice show a slowdown in the rate of increase in IgG2a levels between 6 and 8 days after injection. Challenge with *Salmonella* for a second time on the 16th day resulted again in a rapid increase in IgG2a concentration in all mice after 2 days.

TABLE III  
*Relative Concentrations of IgG2a<sup>b</sup> in Sera from Adult (BALB/c × C57BL) F<sub>1</sub> Mice Treated with 17  $\mu$ g Lipid A/g Body Weight*

Mouse no.	Relative IgG2a <sup>b</sup> concentration	
	At the day of injection	6 days after injection
1	2.07	1.74
2	2.02	2.00
3	2.13	1.75
4	1.47	1.58
5	1.93	1.36

*IgG1<sup>a</sup> + IgG2a<sup>a</sup> Levels After Salmonella Injection into Newborn Mice.*—All mice discussed so far were from matings of the type BALB/c ♀ × C57BL ♂ and were tested for allotype-b (paternal). The ability to be stimulated, however, is not a special property of the C57BL genome. IgG1 and IgG2a concentrations of allotype-a in litters of C57BL ♀ × BALB/c ♂ mice are also enhanced after *Salmonella* injection (Fig. 4). Mice injected 7 days postpartum show measurable IgG levels about 3 days earlier than their noninjected controls. Enhancement of IgG concentration is 2–3-fold after the first, and about 10-fold after the second injection. The relatively high levels are maintained till adulthood.

*Antibody Specificity.*—It has been shown (12) that lipid A when injected in vivo induces antibodies of the IgM class which react with red blood cells of different species. This leads to the question whether the IgG induced by lipid A in the newborn mouse possesses any specificity. The inherent characteristic of the test system is that of a hemolytic assay. If the sera used for inhibition of facilitation contained IgG anti-SRBC they would bind to the cells and, complexed with anti-allotype, lead to further lysis instead of inhibition of lysis. We have not found this effect either in lipid A-injected or *Salmonella*-injected mice. We conclude that the bulk of IgG resulting from stimulation are not directed against SRBC.

Sera from mice with the most enhanced IgG2a<sup>b</sup> levels were tested for antilipid A activity by the method of Galanos (6)<sup>2</sup> with a modification using anti-IgG2a<sup>b</sup> serum to detect this class of antibodies in mice. No antilipid A activity was found in the sera tested. Further, even the mice injected twice with

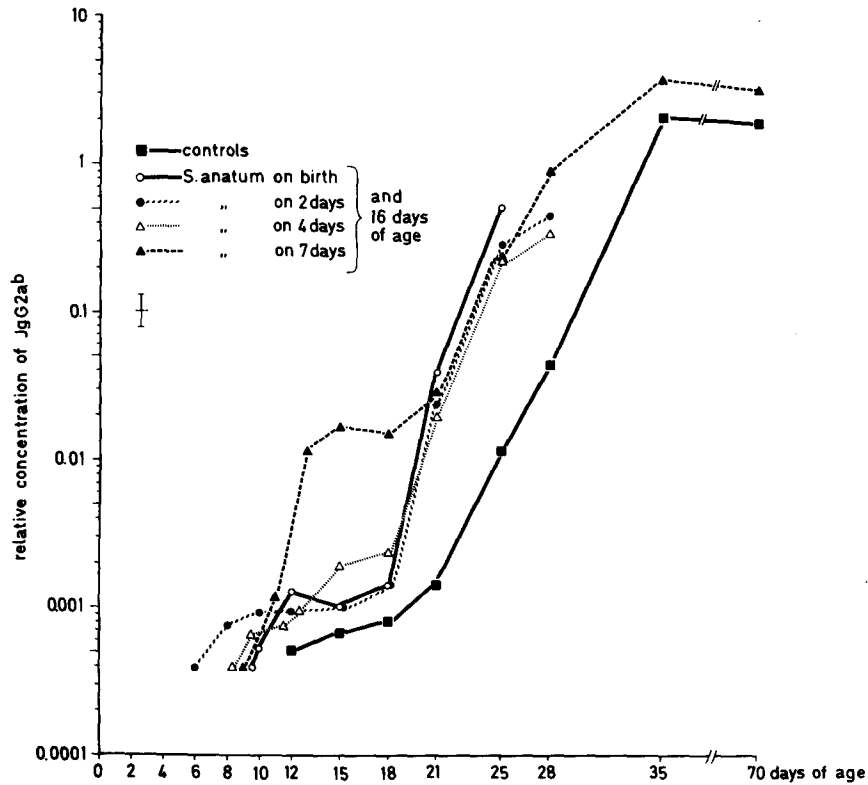


FIG. 3. Relative concentrations of IgG2a<sup>b</sup> in sera from (BALB/c × C57BL/6)F<sub>1</sub> mice treated with 0.03 mg lyophilized *Salmonella anatum*/g body weight on different days (0, 2, 4, or 7) after birth and challenged on day 16. Each point is the mean from values of nine mice. The standard error is 0.114 and is shown by a bar in the graph.

*Salmonella* did not show detectable IgG anti-LPS antibodies by a sensitive hemolytic method (Di Pauli, unpublished observations).

*Spleen Weights.*—In control animals which were injected neonatally either with 2 μg BSA or 100 μg dinitrophenyl-keyhole limpet hemocyanin (DNP-KLH) or were left untreated, the ratio of spleen weight to body weight re-

<sup>2</sup> The lipid A-NaOH for coating SRBC and a positive rabbit antilipid A as a control for the test system was kindly provided by Dr. Chris Galanos, MPI für Immunbiologie, Freiburg, Germany.

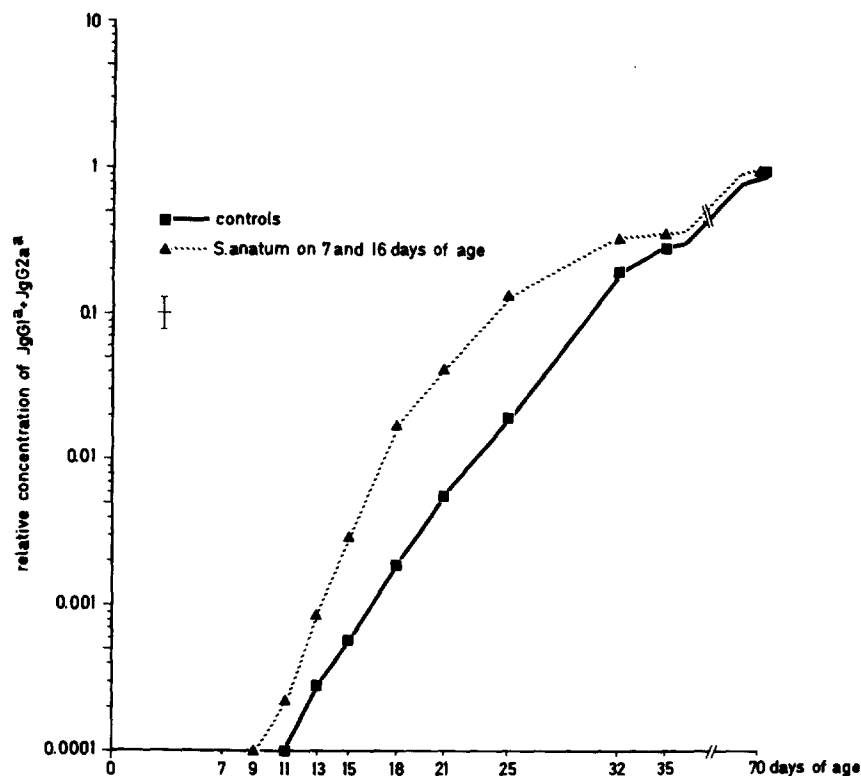


FIG. 4. Relative concentrations of IgG1<sup>a</sup> + IgG2a<sup>a</sup> in sera from (C57BL × BALB/c)F<sub>1</sub> mice, treated with 0.03 mg lyophilized *Salmonella anatum*/g body weight on day 7 and 16 of age. Each point is the mean from values of five mice. The standard error is 0.0857 and is shown by a bar on the graph,

mained nearly constant throughout the time tested (2–13 days of age). After injection of lipid A or *Salmonella*, however, the relative spleen weights increased from the control value of about  $0.6 \times 10^{-2}$  to values twice as high 3–4 days after injection (Table IV) regardless of the absolute age, the age at injection, or absolute spleen and body weights.

#### DISCUSSION

Lipid A has many effects on the immune system. It induces DNA synthesis, IgM synthesis, and secretion in B lymphocytes (2,13). It can also result in escape from tolerance (14,15) and can circumvent the T-cell dependence for certain antigens (16) or trigger immune responses against molecules otherwise not immunogenic (17). All these different activities suggest that the trigger mechanism may be independent of antibody specificities on the lymphocytes.

In previous experiments done *in vitro* (16) IgG induction by LPS has been



TABLE IV  
*Relative Spleen Weights at Different Days after Lipid A or Salmonella Treatment of Neonatal Mice*

Treatment	Days after treatment						
	0	2	3	4	5	6	8
Lipid A		1.1	1.2	1.1	1.1	0.9	0.8
Salmonella bacteria		0.9	1.0	1.1	0.9	0.9	0.8
Control*	0.6	NT†	NT	0.61	NT	0.6	0.5

Values are the ratio of spleen weights (grams) to body weight (grams)  $\times 10^2$  from at least three mice.

\* Either untreated, or treated with 2  $\mu\text{g}$  BSA or 100  $\mu\text{g}$  DNP-KLH at 2 days of age.

† NT, not tested.

obtained only with primed cells from adult mice, and the IgG was directed against SRBC (the priming antigen). No IgG synthesis has been found in normal adult cells in vitro (1). In this report we show that IgG can be induced in mice by lipid A treatment in vivo and that this is possible only in the post-natal period. Since, from the present experiments, we cannot conclude whether this lipid A-induced increase in serum IgG levels is the result of proliferation of producing cells, enhancement of IgG production in preexisting cells, or both, we shall call this increase IgG amplification.

In order to measure the serum IgG concentration in neonatal mice it was necessary to develop a sensitive assay. This technique allows the quantitative determination of paternal allotype in concentrations as low as  $5 \times 10^{-4}$  of normal adult serum. A determination of absolute concentrations of the IgG2a<sup>b</sup> was not possible since no such myeloma protein was available. However, from the known adult levels of IgG2a, 4mg/ml, (18,19) we estimate that the sensitivity of our method is around 2  $\mu\text{g}$  IgG2a<sup>b</sup>/ml serum. By this method we can detect paternal IgG in young mice at around 12 days of age, as compared to 3 wk (19) found with other methods. At that age the relative IgG2a levels are at least  $1.5 \times 10^{-3}$  of adults.

Lipid A or *Salmonella* treatment of (BALB/c  $\times$  C57BL/6)F<sub>1</sub> litters (Figs. 2 and 3) results in a maximum 30-fold amplification of IgG2a<sup>b</sup>. For detection of IgG amplification in (C57BL/6  $\times$  BALB/c)F<sub>1</sub> an antiserum directed against both IgG1<sup>a</sup> + IgG2a<sup>a</sup> classes was used. In this case the maximum increase of both classes together was about 10-fold (Fig. 4) and consequently lower than that for IgG2a<sup>b</sup>. This could be explained if we assume that the class amplified is mainly IgG2a whereas the IgG1 levels are not affected so much. That both allotypes can be amplified suggests that this is not a property restricted to the allotype-b genome.

The increase in IgG levels is rapid and is independent of the age when injections were performed. Irrespective of whether mice were injected at 2, 4, 7, or 16 days of age, IgG amplification was always observed two days after

treatment. The maximal levels reached after stimulation depend on the age at injection. This suggests that the number of cells which can be triggered by lipid A increases in the first days after birth.

Together with the IgG amplification we also observed an enlargement of the spleen. Relative spleen weights were increased to twice those of the uninjected animals, 3-4 days after the treatment. 1  $\mu$ g BSA or 50  $\mu$ g DNP-KLH/g body weight did not affect spleen weights throughout the time tested (Table II). This indicates that lipid A and *Salmonella* stimulate proliferation of cells in the spleen.

We could not find any antibody activity against SRBC, lipid A, or LPS of the IgG class. This does not exclude the presence of small populations of antibodies against these antigens, but at least the bulk of synthesized IgG in the newborn does not react with these antigens. Thus, we assume that a statistical distribution of all specificities is induced.

As has previously been pointed out (17) two possible basic mechanisms can be envisaged which explain the amplifying action of lipid A. They have been discussed in the light of the results obtained with IgM but in principle can be considered valid also for IgG. The interaction of lipid A with the B cell could be a direct single signal which triggers off a phenotypic change in the cells, leading to immunoglobulin production. This would exclude the need for co-operation with any other cell type. The other possibility is a two-signal mechanism; this implies that lipid A by itself is not sufficient for induction of IgG synthesis in B cells, but that an additional specific signal must be provided either by the interaction of antigen with the antibody receptor on the cell surface or by a second cell type.

The possibility that the second signal is provided indirectly through the interaction of B cells with T cells is not ruled out in our system. To test whether the thymus is involved in IgG amplification in the newborn, breeding of (BALB/c  $\times$  C57BL/6)F<sub>1</sub> nu/nu mice is in progress.

Since the in vivo IgG amplification is obtained only in the young mouse, both the single signal and the two signal hypothesis would imply the following possibilities: Cells reactive for lipid A (single signal), for antigen plus lipid A (two signals), or cells mediating the second signal are present only in the post-natal period. If we consider antigens as a second signal there is the possibility that either the antigens or the cells reactive for it are present only in the newborn mouse.

#### SUMMARY

IgG of paternal allotype first becomes detectable in the serum of (BALB/c  $\times$  C57BL/6)F<sub>1</sub> mice between day 12 and 14 after birth and reaches adult levels at an age of 5 wk. Since in mice there is a transfer of maternal IgG molecules through the placenta and via milk, F<sub>1</sub> heterozygous at the allotype locus were used and the concentrations of IgG with paternal allotype were measured.

This was done by a sensitive method capable of detecting IgG concentrations as low as  $5 \times 10^{-4}$  of normal adult serum levels. It is based on the quantitative inhibition of allotype-specific facilitation of hemolysis.

When lipid A or *Salmonella* bacteria were injected into neonatal mice, a stimulation of IgG synthesis was observed. Thus IgG levels were enhanced 10–30-fold compared to the nontreated mice. No increase in IgG levels was obtained in adult mice after treatment with lipid A. Whether the newborns were injected at birth, on day 2, 4, or 7, IgG was first demonstrable in the treated mice at an age of 6–11 days.

The increase in IgG levels was not paralleled by a demonstrable antibody activity against lipid A, SRBC, and LPS. Thus the bulk of newly induced IgG is probably a statistical distribution of different specificities.

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