ONTOGENY OF THE IMMUNE RESPONSE*

II. Correlations Between the Development of the Afferent and Efferent Limbs

By G. M. WILLIAMS,‡ M.D.

(From The Walter and Eliza Hall Institute, Melbourne, Australia)

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In the preceding paper we presented autoradiographic studies of antigen localization patterns in rats of varying ages (1). Newborn rats lack organized antigen-retaining structures in lymph nodes and spleen. Well-defined cortical and medullary antigen retention systems develop in the aortic lymph nodes by the age of 14 to 16 days. At this time less antigen is found diffusely spread throughout the node and localization to segmental cortical areas and medullary macrophages occurs.

The present study attempts to lend significance to the changing patterns of *Salmonella* flagellar antigen distribution during development by correlating the localization of antigen with the subsequent antibody response. We have found that a close temporal relationship exists between the maturation of the antigen-trapping systems and the ability to generate a prompt anibody response.

Materials and Methods

Animals.—Randomly bred Wistar rats were used. In most cases each litter was divided into experimental and control groups and several litters used for a given experiment. Animals were weaned at 4 to 5 weeks.

Technique of Injection and Preparation of Serum.—Polymerized flagellin from *Salmonella adelaide* was the antigen used (2). All animals injected at birth received subcutaneous injections. Originally injections were made into the hind foot pads, but less concentrated batches of antigen necessitated injections under the skin of the back. Both types of injection gave comparable results. All animals were bled from the tail under ether anesthesia. Animals less than 3 weeks of age were bled from the tail under mild suction. Serum was separated from blood after standing at room temperature for 30 min and at 4°C for 4 hr. Complement was inactivated at 56°C for 30 min.

Titrations.—The bacterial immobilization (anti-H) titer of serum samples was determined

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‡ Present address: Department of Surgery, Medical College of Virginia, Richmond, Virginia.

as described previously (2). In essence this method consists of adding a standard concentration of actively motile bacteria to serial dilutions of serum. Microdrops are placed on glass slides under oil and read microscopically for immobilization. The end point is considered to be the highest dilution resulting in significant immobilization and the titer is expressed as the reciprocal of the serum dilution. Most samples were titrated also for their content of antibody resistant to 0.1 M 2-mercaptoethanol (1 hr, 37°C). In our system, most mercaptoethanol-resistant (MER) antibody is IgG and mercaptoethanol-sensitive (MES) antibody is Ig M (3).

Statistical Methods.—All titration data was entered on IBM punched cards and statistical parameters were obtained by computer as previously described (4).

X-Irradiation.—Young animals were aligned in a Perspex box surrounded with 8 cm of packing having the absorbtion characteristics of body tissues. Radiation was generated at 235 kv, 15 ma, half value layer of 1.0 mm of Cu, at a dose rate of 68 rad/min. The beam was vertical, and directed upon the animals which were placed over a lead plate 2.5 cm thick. The standard dose was 450 rad.

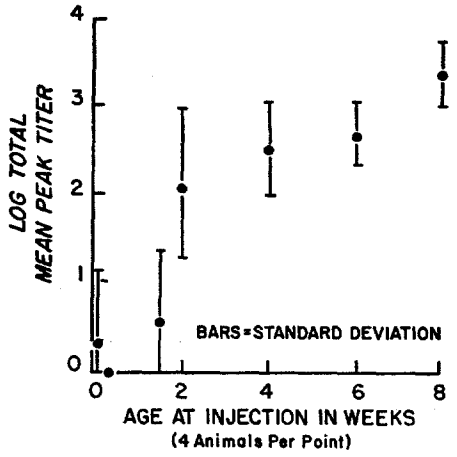

FIG. 1. Antibody response to 100 μg polymerized flagellin given at various ages.

RESULTS

The Relationship Between Age and the Ability to Generate a Primary Immune Response.—Four rats from each of 6 litters aged 0, 2, 10, 16, 28, and 42 days were injected into both hind foot pads with 100 μg of polymerized flagellin. These animals were litter mates of rats injected with radioactive antigen presented in the accompanying paper (1). The mean peak antibody titer increased with age at injection (Fig. 1). Rats injected at or after the age of 16 days produced antibody following a latent period of about 4 days. Animals injected earlier than 2 weeks of age generally failed to produce antibody, or did so in trace amounts following a latent period of some weeks. In this experiment no animal produced detectable levels of antibody prior to 20 days of age regardless

of the age at initial injection. Thus a distinct qualitative change occurred in the response of the lymphoid system at the age of 2 weeks. Interestingly, the rats injected at 2 weeks of age formed MES (IgM) antibody in low titer and for only a few days, rapidly switching to the production of MER (IgG) antibody to much higher titer. Following this time point further maturation produced an increase in the magnitude but not the character of the antibody response.

The Relationship between Age at Primary Antigen Injection and the Induction of Immunological Memory.—Six weeks following primary injection the rats presented above were injected a second time with the same dose of polymerized flagellin. Results of titrations following secondary challenge revealed clear-cut

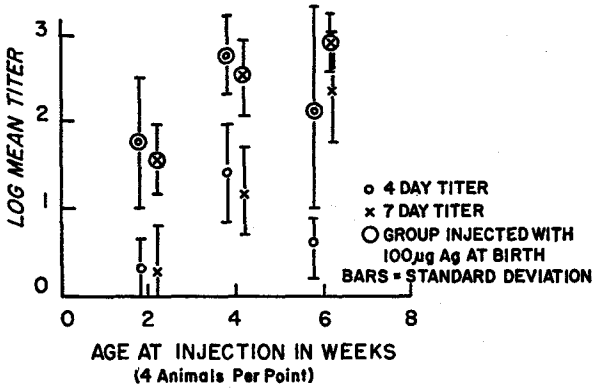

FIG. 2. The effect of a single neonatal antigen injection of polymerized flagellin on a subsequent challenge with the same antigen.

evidence of a secondary response in virtually every animal. Even rats injected at birth which failed to produce a detectable primary response reacted to the secondary challenge at 6 weeks with a more rapid production of higher levels of antibody than control rats receiving a primary injection at age 6 weeks. It appeared that injections of antigen during the first 2 weeks of life were capable of "priming" but not of inducing antibody production.

In order to ascertain when this priming effect was first demonstrable, rats were injected by foot pad with the standard dose of 100 μg of polymerized flagellin at birth and challenged at various ages with a second injection of 100 μg. Littermate control rats received no injection at birth and a challenge of 100 μg of polymer at various ages. In Fig. 2, the results of titrations on serum samples taken 4 and 7 days after challenge are presented. In each case, the circled values, i.e. those from experimental rats, exceeded those not circled; i.e., the control values. Thus, the events of priming must have occurred as early as during the first 2 weeks of life.

The results of a similar experiment are presented more fully in Fig. 3, where rats were given 100 μg of polymerized flagellin either once, at age 16 days, or twice, at age 0 and 16 days. Clearly, the secondary response in these young rats was quite short-lived. In fact, had serum samples only been taken at age 8 weeks one might have concluded that the neonatally injected group displayed partial immunological tolerance.

The Nature of Early Immunological Memory.—MER (IgG) antibody titers were determined in rats injected, at birth and at various later times, with 100

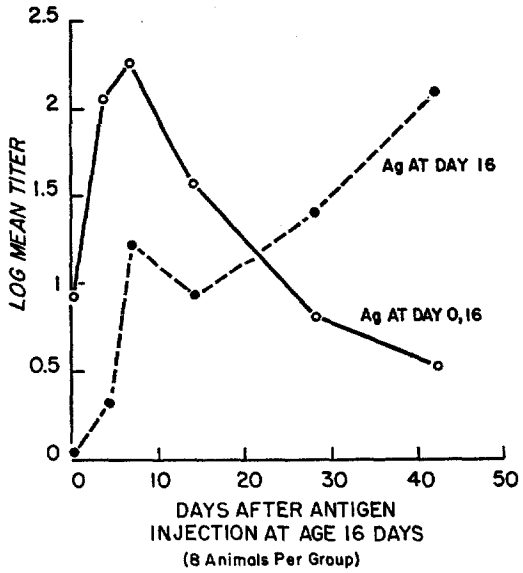

FIG. 3. Effects of neonatal injections of polymerized flagellin on the antibody response to challenge at 16 days.

μg of polymerized flagellin. Rats injected at birth and at either 4 or 6 weeks of age produced IgG antibody somewhat more rapidly than controls that were injected for the first time at these ages (Fig. 4). The peak IgG response was slightly lower in the neonatally injected group. Animals injected at birth and at 2 weeks produced very little IgG antibody (Fig. 4) despite the rapid production of IgM antibody (Fig. 3).

In order to lend statistical confirmation to these findings, all animals injected at days 0 and 14 through 16 with 100 μg of polymerized flagellin were grouped and compared with controls injected at day 14 to 16 alone. For the purposes of comparison the antibody curve was divided into three sections: day 4, representing early IgM production; day 7, peak IgM production; and day 42, peak IgG production. Titers above 20, 40, and 160 were chosen to represent significant

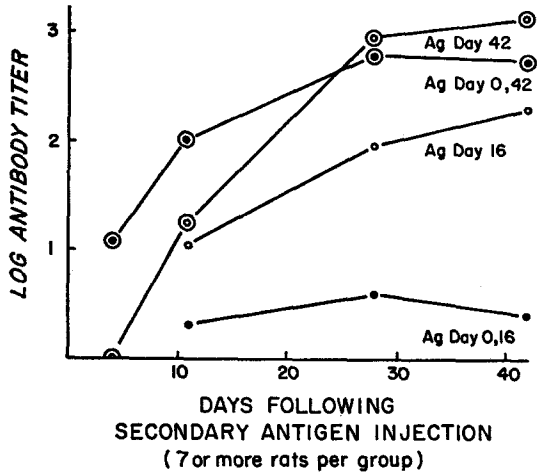

FIG. 4. Effects of neonatal injections on mercaptoethanol-resistant antibody titers following second injections.

TABLE I

Comparison between the Antibody Response to 100 μg of Polymerized Flagellin Given at 14 to 16 Days of Age in Rats Injected and Not Injected Neonatally with 100 μg of Antigen

	Antibody titer at 4 days greater than 20	Ant body titer at 7 days greater than 40	Antibody titer at 42 days greater than 160
Rats injected at day 14 to 16	1*/14‡	5/12	5/5
Rats injected at day 0 and day 14 to 16	19/22	17/18	6/15
Probability of differences occurring by chance (X^2 test)	$P \ll 0.01$	$P \ll 0.01$	$P < 0.05$

* Numerator indicates number of rats responding.

‡ Denominator indicates number of rats tested.

dividing lines for measuring antibody production at these respective time points. The results are presented in Table I. Animals injected at days 0 and 14 to 16 responded in significantly greater numbers during the early IgM phase of antibody production. However, significantly fewer animals injected at these times were able to sustain IgG antibody production. Thus neonatal injections primed animals for a burst of IgM antibody production, but the combination of 0 and 14 to 16 day injections inhibited IgG production.

Additional rats were injected at days 0 and 7 to determine if relative antigen deficiency might be reponsible for the failure of young rats to produce antibody. The results of titrations at day 16 in 15 animals were compared with animals

TABLE II

Comparison of Responses in Rats Injected with 100 μg Polymerized Flagellin on Day 0, 7, 16, and Day 0, 16

Antigen injected at day	No. of animals	No. of animals responding at day 16	Mean antibody titer day 16	Mean antibody titer day 20
0, 16	14	5	12	110
0, 7, 16	15	12	23	356
Probability of differences occurring by chance (X^2 test)		$P < 0.05$	$P = 0.5$	$P = 0.4$

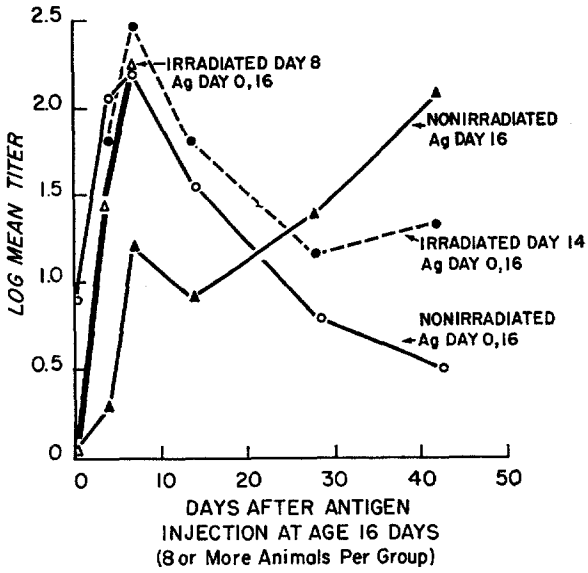

FIG. 5. Effects of X-irradiation on the antibody response of animals primed at birth and challenged at day 16.

injected on day 0 alone (Table II). A significantly greater proportion of animals responded as the result of the additional injection at day 7, but the mean antibody titer was still very low. A third injection of 100 μg of antigen was given at day 16 and again there was no significant difference 4 days after the challenge. Thus the additional injection of antigen at day 7 had a marginal effect only in causing increased antibody production.

The Age at which "Memory Cells" First Appear.—An attempt was made to pinpoint the time of appearance of primed cells by utilizing the well-known fact (5) that immune responses dependent on the presence of primed cells can occur after substantial whole-body irradiation.

Three litters of rats were divided into two groups, each of which received 100 μg of polymerized flagellin subcutaneously on the day of birth. One group was given 450 rad whole-body X-Irradiation at age 7 days and the other at age 14 days. Both groups were challenged with 100 μg of antigen at 16 days of age. The results of antibody titrations (Fig. 5) revealed no inhibition of antibody formation by irradiation either at day 7 or day 14. The early antibody response was a vigorous and characteristic IgM secondary response. For unknown reasons, the rats irradiated at day 8 had a high mortality at 2 and 3 weeks postirradiation. Consequently, the unaltered early response to secondary challenge is the more remarkable. It appears as if neonatal injections of antigen induced early synthetic steps prior to 1 week of age which created certain primed cells capable responding in heightened fashion to antigen despite prior irradiation.

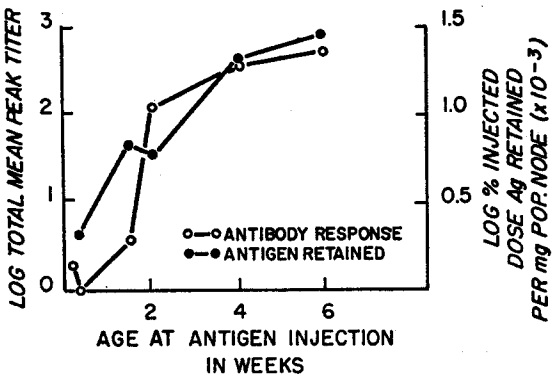

FIG. 6. The relationship between the amount of antigen retained 6 days after a primary injection and antibody titers of littermates injected identically.

DISCUSSION

The studies on the development of the afferent and efferent limbs of the immune reaction have demonstrated certain striking temporal correlations. At the age of 10 days antigen was first found localized in organized structures, and at the age of 14 to 16 days these structures in the cortex of lymph nodes first became recognizable as the *Anlagen* of the primary lymphoid follicles (1). Also at the age of 14 to 16 days the rat first produced a significant and typical primary antibody response (Fig. 1). After the age of 2 weeks both the amount of antigen retained in lymphoid tissues and the amount of antibody produced increased steadily. The correlation between antigen retention and antibody production is illustrated in Fig. 6, which is based on data from both of the present papers. It should be emphasized that the increase in antigen retention demonstrated is not the result of increased lymphoid mass but rather a more basic change increasing the amount of antigen retained per unit mass of lymphoid tisuse. Thus both

qualitative and quantitative steps in the development of antigen-capturing structures find their parallel in the development of the primary antibody response.

It was originally anticipated that a neonatal injection of 100 μg of polymerized flagellin might induce some degree of tolerance. However, the single injection at birth produced a complex immunological state characterized by the following reactions: little or no primary antibody production (Fig. 1); clear-cut IgM secondary responses to challenge injections at the age of 2, 4, or 6 weeks (Fig. 2); equally clear-cut depression of the IgG antibody response to challenge at age 2 weeks (Table I). Thus neonatal injections initiated but failed to sustain antibody production and produced a state of 19 S "memory" and 7 S "tolerance" at the 2 week time point. These findings posed two important questions: (*a*) Is cellular differentiation arrested at the primed cell stage in immature animals, and if so what is the mechanism? (*b*) What is the relationship between priming, arrest of further differentiation, and the ease of tolerance production?

In attempting to answer the first question we found the scheme of hypothetical cellular differentiation proposed by Sercarz and Coons (6) quite helpful. On the basis of transfer experiments these investigators postulated the need for cells during various phases of differentiation to have contact with antigen.

$$\underset{\text{"X cell"}}{\text{Primitive competent cell}} + \text{Ag} \rightarrow \underset{\text{"Y cell"}}{\text{primed cell}} + \text{Ag} \rightarrow \underset{\text{"Z cell"}}{\text{plasma cell}}$$

If maturation of primed cells were arrested by a relative antigen deficiency as suggested by the previous paper (1), then an additional injection of antigen should be capable of stimulating the primed "Y cells" to antibody forming "Z cells." Such an experiment was conducted, (Table II) and rats injected at day 0 and 8 produced no greater levels of antibody by day 16 than animals injected on day 0 alone. Thus gross antigen deficiency alone cannot explain the arrest in differentiation.

However, it is possible that very few competent "X cells" existed during the first week of life, and that priming occurred only after the age of 1 week regardless of when antigen was givem. To test this assumption rats injected at birth were irradiated at day 7 with 450 rad. Based on the apparent sensitivity of the primary, but not the secondary response to irradiation prior to antigen administration (5), we anticipated that responses dependent on X cells, but not those dependent on Y cells, would be damaged by irradiation. Thus if a large population of Y cells existed prior to irradiation at 7 days, irradiation might have little effect on the response to challenge at 16 days. This was our finding. It could be argued that in fact this dose of irradiation might not have suppressed a primary IgM response (7–9), but extensive experiments in this laboratory have demonstrated that in our system, this phase is just as radiosensitive as the IgG phase

(10). Our results are explained best by the postulate that Y cells were present already at 7 days of age and that these failed to continue differentiation into antibody-forming cells in the neonatal lymphoid environment. This is compatible with certain cell transfer experiments (11–14) which have shown newborn animals to be inadequate hosts for primed cells under certain circumstances. The causative mechanism of such a maturation arrest remains obscure.

The relationship existing between this apparent block in cellular differentiation and the ease of tolerance formation in immature animals may prove of interest. Previous work from this laboratory has demonstrated that twice weekly intraperitoneal injections of polymerized flagellin can lead to virtually complete tolerance (15). Yet, when polymer was injected, less frequently in the newborn period, e. g. on days 0, 8, and 16 (Table II), considerable IgM antibody formation ensued. It has been suggested by Rowley and Fitch (16, 17) that cells from young animals in a critical stage of differentiation may be unusually susceptible to suppressive effects of a pulse of antigen. Our findings are consistent with this view. It is possible that the conversion of Y cells to Z cells is blocked in newborn animals, and a single neonatal injection could thus lead to a build-up of Y cells. These might be susceptible to suppression by antigen injected 3, but not 8, days later. However, we cannot exclude the possibility that X cells are converted to the tolerant state directly (18), nor do we know whether cell death or some intracellular change is involved. Sterzl (19) has also drawn attention to the relationship between priming, IgM antibody production, and tolerance induction in newborn animals. Further work is in progress on the different effects of neonatally injected monomer and polymer, and this will be reported separately. However, it is clear that once antigen-trapping mechanisms of an effective sort develop for polymerized flagellin at 10 to 14 days of age, primary responses with IgM to IgG transitions of typical adult pattern are the rule. It is likely that antigen processing defects account at least in part for the various anomalous reactivities of newborn animals.

SUMMARY

The development of the ability of young rats to generate a prompt primary antibody response to polymerized flagellin, with IgM to IgG transition, is correlated in time with the development of structures in the cortex of lymph nodes that localize antigen to spherical areas which subsequently become primary lymphoid follicles.

Throughout development the increased magnitude of the antibody response parallels the increased ability of lymphoid structures to retain antigen.

During the first week of life primitive lymphoid tissue appears capable of undergoing the initial steps in differentiation toward antibody production in response to neonatal injections of polymerized flagellin. However, further maturation appears to be blocked resulting in a complex immunological state at the

age of 2 weeks characterized by increased IgM and decreased IgG antibody response to antigenic challenge at this time.

The possible relationship between the block in cellular differentiation toward antibody formation and the ease of tolerance induction is discussed.

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