Immunologic Responses of Neonatally Thymectomized Rats to Sheep Red Blood Cells and to Flagellin from *Salmonella adepaide*[1] (35972)

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(Introduced by S. Raffel)

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Conditions for demonstrating the dependence of the immune response upon an intact thymus gland vary, depending upon the species of animal, the age of the animal when the thymus is removed, as well as what antigen is used (1). Neonatally thymectomized mice show a depressed serologic response to immunization with sheep red blood cells (2). Unlike the case in the mouse, neonatal thymectomy in rats does not depress the serologic response to sheep red blood cells (3). Also unlike events in the mouse, neonatal thymectomy of the rat does depress the serologic response to flaegllin, the flagellar protein obtained from *Salmonella adelaide* (3, 4). The purpose of the present study was to investigate at the cellular level the effect of neonatal thymectomy upon the response of the rat to a primary challenge with sheep red blood cells, as well as to polymerized flagellin (POL) from *S. adelaide*. The cellular response to sheep red blood cells was followed using a modification (6) of the Jerne technique, and the cellular response to POL was followed using Diener's technique (7) for detecting lymphoid cells showing bacterial adherence. These cellular responses were correlated with the corresponding serologic responses.

An unexpected observation was made: although the serologic response to POL is markedly impaired in neonatally thymectomized rats, the number of lymphoid cells in these animals showing an acquired specificity for the antigen POL was not markedly depressed when compared with sham-thymectomized control animals giving good serologic responses. This finding suggested that, for this antigen in the rat, neonatal thymectomy may not interfere with antigen recognition, but may interfere with the secretion of avid antibody from cells following antigenic stimulation.

Materials and Methods. Wistar and Lewis rats of both sexes were used. Thymectomy was performed within 24 hr of birth. Rats were anesthetized by maintaining them at −18° for approximately 10–15 min. The thymus was exposed by excising the anterior end of the sternum as well as the sternal portion of the two anterior-most ribs. Ablation of the gland was accomplished by suction through the tip of a small glass pipette attached to a faucet aspirator. The skin was approximated with silk sutures and the animal was returned to its mother. Sham-thymectomized rats were subjected to the same anesthesia and operative procedures to expose the thymus which was not removed.

The primary criterion for the success of thymectomy was the absence of any histological evidence of thymic tissue in the mediastinum at the time the rat was sacrificed. A block of mediastinal tissue was sectioned, and approximately every fifth section was mounted and stained.

An additional criterion used for the classification of an experimental animal as completely thymectomized was that it gave a clearly depressed serologic response to POL.

Sheep red blood cells used for antigenic stimulation were given intraperitoneally in 0.5 ml as a 20% suspension in physiologic saline.

Polymerized flagellin (POL) used for antigenic stimulation was obtained from *Salmon-*

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ella adelaide (035, fg) by Ada *et al.* (5). Twenty-five micrograms of POL in 0.5 ml of physiologic saline were injected intravenously.

Humoral antibody to sheep red blood cells was determined by the technique of hemagglutination. Twofold dilutions of serum were prepared; and to these, an equal volume of a 1% suspension of washed sheep erythrocytes in saline was added. These mixtures were examined after 1.5–2 hr at room temperature. The end point was that dilution of serum that agglutinated approximately half of the red cells as determined by the pattern of settled erythrocytes. Titer was expressed as the reciprocal of this dilution. In all titrations a standard control serum of high titer was included.

The titer in serum of antibody to flagellin was determined by the technique of bacterial immobilization as described by Ada *et al.* (5). Bacteria utilized in this technique were *Salmonella derby,* containing H antigens f and g in common with *S. adelaide.*

Mercaptoethanol treatment of serum consisted of mixing a 1:5 dilution of serum in saline with 2-mercaptoethanol (2-ME) to give a final concentration of 2-ME of 0.1 *M.* This mixture was maintained at 37° for 60 min. Titers were determined at the same time on the same sera treated and untreated with 2-ME.

The number of lymphoid cells producing hemolytic antibody to sheep red blood cells [plaque forming cells (PFC)] was determined by Cunningham and Szenberg's modification of the Jerne technique (6). In this procedure lymphoid cells from the spleen of the rat and red blood cells from the sheep are suspended together in liquid medium and placed by capillary action in a chamber made by closely approximating two glass microscope slides. Pooled guinea pig serum was used as a source of complement.

The number of lymphoid cells producing antibody to flagellin was determined by a technique developed by Diener (7). Briefly, this technique involves mixing a single-cell suspension of lymphoid tissue with actively motile *S. derby,* sharing H but not O antigens with *S. adelaide.* Lymphoid cells supposedly elaborating antibody directed against the shared specific flagellin become coated with *S. derby.* Aliquots of this suspension containing lymphoid cells and Salmonella are then plated in agar. The petri dishes are incubated at 37°, but growth is interrupted when large bacterial colonies evolving from lymphoid cells showing immune adherence of *S. derby* can still be clearly differentiated from the very small colonies arising from individual bacteria.

Results. I. Response of neonatally thymectomized rats to immunization with sheep red blood cells. All animals used in these experiments were Wistar rats. They were immunized at 4 weeks (± 2 days) of age and killed 4, 7, or 28 days later. Prior to death, serum was collected to determine its hemagglutinating titer, and spleens were removed to determine the number of hemolytic PFC. Results of these experiments are shown in Table I.

Although at 4 days after immunization, the sera of completely thymectomized rats have lower titers of hemagglutinating antibody than do sham-thymectomized control animals, the difference between the mean values for these two groups then disappears. At 7 days and 28 days after immunization the serologic titers for antibody directed against the sheep red blood cells were comparable for all rats whether they were completely thymectomized, incompletely thymectomized, or sham thymectomized. Thus, it is clear that under these conditions neonatal thymectomy does not depress either the serologic or cellular responses of the rat to immunization with sheep red blood cells, although it seems to delay somewhat the appearance of these immunologic responses.

II. Responses of neonatally thymectomized rats to immunization with polymerized flagellin. Both Wistar and Lewis rats were utilized in these experiments. The data presented here were obtained from rats challenged intravenously at 8 weeks of age with 25 μg of polymerized flagellin. At 5, 14, or 21 days following immunization, the immunized rats were bled to obtain sera for determining titers of antibody participating in bacterial immobilization. The animals were then killed; and the spleen and/or lymph nodes

TABLE I. Effect of Neonatal Thymectomy Upon the 19S Hemolytic Plaque Forming Cells in the Spleen and the Hemagglutinating Titer of Rats Immunized at 4 Weeks of Age with Sheep Red Blood Cells.

Status of rats	Day:	(Mean no. of PFC per 10^6 nucleated cells of spleen)/(mean hemagglutinin titer of serum); days after immunization with sheep red blood cells: 0	4	7	28
Normal		2/<5 (12)[a]			
Sham thymectomized			390/160 (5)	40/410 (4)	15/360 (5)
Incompletely thymectomized			220/40 (10)	55/510 (3)	15/430 (3)
Completely thymectomized			70/10 (4)	100/370 (3)	15/270 (3)

[a] No. of rats in group.

were removed to determine the number of lymphoid cells showing the immune adherence of bacteria. Results of these experiments are shown in Tables II and III.

The serologic titers of rats completely thymectomized at birth are markedly depressed when compared with sham-thymectomized control animals, as shown in Table II. Incompletely thymectomized rats having small remaining remnants of thymic tissue usually respond well serologically to flagellin. It is therefore clear that complete neonatal thymectomy very markedly affects the serological response to polymerized flagellin as measured by bacterial immobilization. Comparable titers were obtained when antiflagellar antibody was determined by use of a hemagglutinating technique employing tanned red blood cells (kindly done by Mrs. Merril Rowley).

There is a transient small rise of immobilizing antibodies seen on day 5 following immunization of completely thymectomized rats, but this has essentially disappeared by 14–21 days. The possibility that this early transient rise in antibody titer was 2-mercap-

TABLE II. Serologic Responses of Rats Completely Thymectomized (Tx), Incompletely Thymectomized (Inc. Tx), and Sham Thymectomized (STx) at Birth and Immunized When 8 Weeks Old with 25 μg of Polymerized Flagellin from *S. adelaide*.

Status of rat	Mean titer of bacterial immobilizing antibody in sera at the following days after immunization: Preimmun.	Days: 5 Saline	5 2-ME[a]	14	21	28
Tx (9)[b]	<2	130 (1 rat =960)	15	55 (6 rats =<5)	100 (6 rats =<10)	100 (6 rats =10 or less)
Inc. Tx (6)	<2	450	25	555	1305	1545
STx (6)	<2	230	90	470	730	1215

[a] 2-ME = serum treated with 2-mercaptoethanol.

[b] No. of rats in group.

TABLE III. The Effect of Neonatal Thymectomy upon the Serologic and Cellular Response of Lewis Rats to Immunization with 25 μg of Flagellin from *S. adelaide* at 8 Weeks of Age.

	Days after injection of flagellin											
	0			5			14			21		
Status of rats	Serum titer[a]	ICA cells/ spleen[b]	ICA cells/ 10^6[b]	Serum titer	ICA cells/ spleen	ICA cells/ 10^6	Serum titer	ICA cells/ spleen	ICA cells/ 10^6	Serum titer	ICA cells/ spleen	ICA cells/ 10^6
Non operated; received no flagellin (6)[c]	<5	10	0.6									
Sham thymectomized;												
received no flagellin (4)	<5			<5	2.0×10^3	2				5	1.5×10^3	5
received flagellin (13)	<5			680	4.7×10^4	115	1280	8.8×10^4	290	1730	1.7×10^4	53
Thymectomized; received flagellin (8)	<5			22	1.4×10^4	65	50	1.5×10^4	70	10	2.7×10^3	18

[a] Mean titer of all sera for group at any one interval after immunization.

[b] Av. no. of lymphoid cells showing immunocytoadherence (ICA) of Salmonella per spleen or per 10^6 nucleated white blood cells in spleen.

[c] No. of rats in group.

toethanol-sensitive was investigated. Sera from 7 completely thymectomized rats immunized with POL at 8 weeks of age and showing this early transient rise in titer were treated with 2-mercaptoethanol. These results are also shown in Table II. It is clear that one cannot conclude that this early transient reponse is entirely 2-mercaptoethanol-sensitive antibody.

The number of lymphoid cells that acquire specificity for the flagellar structure of Salmonella possessing H antigens, fg, markedly increases following immunization with polymerized flagellin. Using the technique described by Diener (7) for determining the number of lymphoid cells showing immune adherence of Salmonella, there is approximately a 3 log increase of such cells in the spleen within 5 days after challenge as shown in Table III. By 14 days, this has increased for sham-thymectomized rats to a 4 log difference. It then falls, although the titers of circulating humoral antibody continue to rise. For the data in Table III at any one interval following immunization, all animals were sacrificed on the same day, and the lymphoid cell suspensions were assayed under identical conditions. At 5 days after challenge, a completely thymectomized Lewis rat with a serum titer of 7.5 had 60 reactive lymphoid cells/10^6 nucleated spleen cells, while a sham-thymectomized rat with a bacterial immobilizing titer of 1280 had 90 reactive lymphoid cells/10^6 nucleated spleen cells. This dichotomy between serologic response and numbers of cells showing immune adherence is even more striking at 21 days.

In some experiments, all of the accessible lymph nodes were removed from the rat, pooled, and disrupted to make a cell suspension which was assayed by the technique of Diener in parallel with the cell suspension obtained from the spleen of the same animal. These results were also compared for animals immunized 7 days previously by the intravenous or the intraperitoneal route. The numbers of cells acquiring specificity for flagellin (per 10^6 nucleated lymphoid cells) were the same whether obtained from the spleen or from pooled lymph nodes. They were also the same whether the antigen was given intravenously or intraperitoneally.

As shown in Table III, sham-operated but non-stimulated control rats of the same strain and age maintained under identical conditions acquire some antigen-adherent cells as they age, although they acquire no detectable immobilizing antibody in their sera. Using Diener's technique, no antigen-adherent lymphoid cells could be found in the spleens of newborn rats, nor in the spleens of young (50 g) rats, but a low background was detected as the normal rats aged.

Therefore, it is clear that, although neonatal thymectomy of the rat reduced its ability to respond to immunization with flagellin by producing serum antibody capable of immobilizing appropriate Samonella, it does not reduce the number of lymphoid cells that acquire a specificity for this antigen as measured by immune adherence of the bacteria to these lymphoid cells.

Discussion. The neonatally thymectomized mouse shows a depressed serologic response to sheep red blood cells (2), but not to flagellin from *S. adelaide* (4). On the other hand, the neonatally thymectomized rat has a normal serologic response to sheep red blood cells, yet it has a markedly depressed response to this flagellin (3). The reasons for such divergent results are not clear.

The observation that the depression of the serum antibody response to flagellin is more impressive at 14 days compared with 5 days after immunization is consistent with similar studies recently reported by Davies *et al.* (8) for mice thymectomized at birth and immunized with flagellar antigen from *S. typhi.* Davies *et al.* (8) also were not able to separate clearly the antibody appearing in the thymectomized mice into IgM and IgG antibody, but could conclude that these mice had a quantitative deficiency of IgG antibody. Thus, it can be stated that, for those flagellar antigens that are thymus dependent, the depression of the serologic titer is more impressive late in a primary response, although it is apparent early.

The bases for the assay techniques used in this study for detection of antibody production at the cellular level differ. The hemolytic plaque in the modified Jerne technique re-

quired that the cell producing antibody *secretes* sufficient antibody to diffuse radially and sensitize adjacent red blood cells. On the other hand, the antibody-producing cell detected by immune adherence may reflect specific antibody on its surface, without any requirement that the cell secretes sufficient antibody to diffuse into the surrounding medium. A more appropriate assay in the red blood cell system to equate with the lymphoid cell showing the immune adherence of Salmonella would be the lymphoid cell showing rosette formation.

An interesting finding in these studies is the discrepancy between the serological and the cellular response in neonatally thymectomized rats immunized with flagellin. Sham-thymectomized rats responded to immunization with good Salmonella-immobilizing titers in their sera, as well as with a pronounced increase in numbers of lymphoid cells showing adherence of Salmonella. In contrast, the completely thymectomized rats failed to respond with titers of immobilizing antibody in their sera, yet these same animals had almost as many lymphoid cells showing adherence of Salmonella as the immunized sham-operated controls. Thus, although the thymectomized rats could not produce measurable serum antibody, some of their lymphoid cells were clearly capable of acquiring surface reactivity for the flagellar antigen. Such cells are not present in the newborn or young rat, are found in small numbers (1–2 per 10^6) in nonstimulated normal adult rats, and appear in large numbers after immunization.

The appearance of lymphoid cells with acquired surface specificity for flagellin in immunized animals that respond poorly in production of detectable serum antibody suggests a delayed hypersensitivity response. However, it has been established for some time that neonatally thymectomized rats show a suppression of immune phenomena of the type attributed to delayed hypersensitivity, including skin sensitization to tuberculin or to bovine serum albumin, experimental allergic encephalomyelitis, and skin homograph rejection (9, 10). Little work has been done, to date, on the induction in the rat of delayed hypersensitivity to flagellin. However, Parish (11) is currently finding that rats develop *no* evidence of delayed hypersensitivity to polymerized flagellin as indicated by swelling of the foot pad following injection of this antigen. He finds that monomeric flagellin, however, can induce weak delayed hypersensitivity reactions. Thus, although one cannot state with certainty that the lymphoid cells showing bacterial adherence in the Diener assay are not associated with delayed hypersensitivity, there is no evidence now available to implicate them in such a role.

Lymphoid cells showing immune adherence of Salmonella appear, under phase microscopy, to be morphologically identical whether they come from a thymectomized rat with a markedly depressed serological response or from a sham-thymectomized rat with a high serologic titer. Studies in microdroplets of isolated single lymphoid cells showing adherence of Salmonella might indicate whether such cells from thymectomized rats contain expected amounts of unreleased avid (immobilizing) antibody as compared with cells from sham-thymectomized rats.

These findings are not inconsistent with the recent evidence presented by Miller and Mitchell (12) that some of the antigen-reactive cells and the antibody-forming cell precursors are of two different cell lineages. The lymphoid cells showing immunocytoadherence in the Diener assay could, in the thymectomized rats, represent the antigen-reactive cells of thymic origin that are themselves incapable of secreting antibody. If this is so, then they must in some way be defective in their required interaction with antibody-forming cell precursors of bone marrow origin. Against this possibility, however, is the fact that these rats were apparently completely thymectomized at birth. Or, it is possible that the cells showing immunocytoadherence are those of bone marrow origin but secreting antibody of low avidity that is not efficiently detected serologically. If of bone marrow origin, it could be also that these cells are synthesizing less antibody per cell or not releasing synthesized antibody. Either of the latter possibilities seem inherently less likely than the secretion of antibody of low

avidity. Perhaps the most exciting interpretation of the increase in lymphoid cells showing immunocytoadherence in thymectomized animals lacking immobilizing antibody in their sera would be that these represent bone marrow-derived antibody-forming cell precursors that increase in number as a specific response to the immunization with flagellin. Miller and Mitchell (12) propose that there are both thymus-derived and bone marrow-derived unispecific cells of the antigen-reactive type. If the thymus-derived cells possessing recognition units on their surface respond with blast transformation and clonal expansion when they come in contact with antigen, why should one not expect unispecific bone marrow-derived cells also to respond with clonal expansion in the presence of antigen? In the case of a thymectomized animal no thymus-derived cells would be present to stimulate the maturation of these antibody-forming cell precursors of bone marrow origin into antibody-secreting cells.

Summary. Rats thymectomized at birth and immunized with sheep erythrocytes at 4 to 8 weeks of age showed cellular and serologic responses comparable with normal or sham-thymectomized rats. However, thymectomized rats immunized at 4 weeks of age with flagellin from *S. adelaide* showed a markedly impaired serologic response, while sham-thymectomized rats had high titers of immobilizing antibody in their sera. Despite the impaired serologic response to flagellin, thymectomized rats acquired nearly equal numbers of lymphoid cells with specificity for flagellin as measured by adherence to their surfaces of viable bacteria possessing flagellar antigens in common with the flagellin used for immunization. This work implies that neonatal thymectomy in this antigen system may interfere with the synthesis of specific antibody rather than antigen recognition following immunization. Although the origin and role of these lymphoid cells showing immunocytoadherence has not been clarified, these data may represent evidence for the antigen-stimulated proliferation of bone marrow-derived antibody-forming cell precursors in the absence of thymus-derived cells.

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