Antigen-Induced Proliferative Response to Murine Thymus Cells in Vitro

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Thymus cells from 5- to 6-week-old normal (unimmunized) BALB/c mice showed an increased incorporation of \(^{3}H\)thymidine in the presence of 2,4-dinitrophenyl-bovine serum albumin, fluorescein-bovine serum albumin, and bovine serum albumin (BSA) in tissue culture. The concentrations of antigen (BSA and haptenated proteins) required for stimulation were approximately 25- to 50-fold higher than those of the nonspecific mitogen, concanavalin A. In contrast to the stimulation by concanavalin A, which was maximal at 24 to 72 h, the stimulation by antigen was most marked earlier in the culture period (6 to 24 h). The BSA response was diminished to a statistically significant degree (especially at low BSA concentrations) in thymocytes from animals injected 72 h previously with BSA, indicating that the stimulation is immunologically specific.

Although there is no doubt that peripheral thymus-derived lymphocytes recognize and respond to antigen, it has not been easy to determine whether this capability is developed before or after they leave the thymus. Lawrence and his colleagues (16) have studied antigen binding in mouse thymocytes and have demonstrated that a significant percentage of cells bind 2,4-dinitrophenyl proteins, but the functional significance of the binding has not been proven. Chiller et al. found that thymocytes from mice injected with monomeric human immunoglobulin G specifically lost their capacity to act synergistically with normal bone marrow cells when transferred to irradiated, antigen-injected syngeneic animals (6), suggesting that antigen recognition can take place in the thymus itself. Attempts to use normal thymus cells directly (without passage through an irradiated syngeneic host) to reconstitute the primary in vitro anti-sheep erythrocyte response in bone marrow-derived mouse splenocytes frequently have failed (9); successful experiments have also been reported (9, 14). An in vivo deoxyribonucleic acid (DNA) synthetic response to antigen (8) has been shown in mouse thymus cells that have been transferred into syngeneic lethally irradiated animals (12, 15). These observations suggest that thymocytes can be stimulated metabolically by antigen, although because of possible problems in interpretation in the in vivo setting, alternative explanations are possible, and the functional significance of the response in terms of the generation of cells responsible for cellular immunity and immunological memory is uncertain.

Because of the importance of being able to study antigen-thymocyte interactions in better defined systems, we have evaluated the DNA synthetic response of isolated mouse thymus cells to antigen in tissue culture. With a similar system (S. A. Eisen, L. R. Lyle, and C. W. Parker, Fed. Proc., 30:649, 1971), we have previously shown that thymus cells from several strains of untreated mice respond with increased incorporation of \(^{3}H\)thymidine when mixed with histoincompatible thymus cells (17). In this report, we describe increased levels of \(^{3}H\)thymidine uptake in thymus cells incubated with protein antigens or hapten-protein conjugates. We will further show that the responses to antigen are reduced in cells from animals preinjected with homologous antigen, indicating that the response is immunologically mediated. Portions of this work have been described briefly elsewhere (L. R. Lyle, S. A. Eisen, and C. W. Parker, Fed. Proc., 31:796, 1972).

MATERIALS AND METHODS

Antigens. 2,4-Dinitrophenyl (DNPh) bovine serum albumin (BSA) was prepared by the Eisen method (10). The material was concentrated by pervaporation and dialyzed against 0.15 M NaCl and 0.01 M phosphate, pH 7.3 (PBS). Fluorescein bovine gamma globulin (F\(_{G}\)yG) was prepared by the method of Nairn (21), but with a 10-fold greater molar excess of fluorescein isothiocyanate. After elution from Sephadex G-25, the conjugated protein was concentrated by pervaporation and dialyzed against PBS. Con-
canavalin A (con A) was the generous gift of G. Kobayashi of the Washington University School of Medicine. Five-times recrystallized BSA was obtained from Pentex Division of Miles Laboratories, Kankakee, Ill., and was dialyzed extensively against PBS before use. Polyvinyl pyrrolidone 360 was obtained from Sigma Chemical Co., St. Louis, Mo. Keyhole limpet hemocyanin was obtained from the Pacific Biomarine Supply Co., Venice, Calif. It was stored in ammonium sulfate solution and prepared according to Campbell et al. (5). Lipopolysaccharide B endotoxin from Escherichia coli 0127:B8 and phytohemagglutinin were purchased from Difco Inc., Detroit, Mich. The proteins were dissolved in PBS and endotoxin was dissolved in water. The proteins and the endotoxin were dialyzed versus PBS and sterilized by membrane filtration (Millipore Corp.).

**Animals.** Nonlitter-mate BALB/c male mice (5 to 6 weeks old) were obtained from the Jackson Laboratories, Bar Harbor, Me.

**Sera.** Rabbit and human sera were obtained aseptically from the blood of healthy adult donors. Guinea pig, fetal calf, and BALB/c mouse sera were obtained commercially from Microbiological Associates, Bethesda, Md., Gibco Biologicals, Grand Island, N.Y., and Jackson Laboratories, respectively. All sera were heated at 56°C for 30 min. BALB/c mouse serum was sterilized by membrane filtration (Millipore Corp.).

**Culture methods.** Thymuses were removed by an aseptic technique and pooled, with care being taken to avoid the inclusion of mediastinal lymph nodes. The cells for each experiment (pooled from five to ten animals) were placed in vessels containing PBS. A single-cell suspension was prepared by passing the organs through a stainless-steel wire cloth and then by filtering them through a 0.3-g segment of PBS-soaked rayon in a small funnel. After counting, the cells were diluted to 1.5 x 10^6 cells/ml in tissue culture medium 199 (North American Biologicals, North Miami, Fla.), buffered to pH 7.3 with 0.02 M Tris (hydroxymethyl)-aminomethane-hydrochloride, containing 10% by volume heated mouse or human serum. Volumes of 1 ml were added to three replicate culture tubes (Falcon 2005) containing 0.04 ml of antigen, mitogen, and their dilutions or buffer. Either at this time or at some interval prior to culture termination, 0.35 µCi of [³H]thymidine (New England Nuclear Corp., 6.9 Ci/mmol) was added to the culture tubes. The incorporation of [³H]thymidine into DNA was determined by isolating the cell pellets by centrifugation and by precipitating the counts incorporated with 6% trichloroacetic acid or by processing on a Millipore 3025 sampling manifold. The precipitate (or filter pad) was solubilized in 0.5 ml of NCS (Amersham/Searle) and was counted by liquid scintillation. The magnitude of the response to antigen is presented in most cases as the stimulation ratio, i.e., the counts per minute of [³H]thymidine incorporated in the cells in the presence of antigens or mitogen divided by the counts per minute of [³H]thymidine incorporated by the cells incubated in the presence of an equal volume of PBS. Counting periods were 4 min. in all cases, and the data presented are counts per minute. In all cases, PBS-containing control tubes constituted not less than 15% of the experiment (usually a total of 12 control tubes scattered randomly throughout the experiment).

To study the effect of exposure to antigen in vivo on thymus cell responses, animals were injected with ultracentrifuged BSA. In preparing the antigen for injection, BSA was dissolved in PBS to a concentration of 20 mg/ml. The concentrated BSA solution was centrifuged at 40,000 rpm in a Beckman Spinco L-2B ultracentrifuge for 150 min with the Ti-65 head. The top quarter of the material in the tube was removed with a pasteur pipette and sterilized by filtration. After determination of the protein concentration (based on absorbancy at 280 nm), the solution was diluted to 2.5 mg/ml in PBS. One milliliter was injected intraperitoneally into ten 5- to 6-week-old BALB/c mice. Ten control animals of the same age received comparable volumes of PBS. Animals were sacrificed 72 h later.

**Endotoxin.** Protein preparations were assayed for pyrogenicity in rabbits and for agglutinating activity in the limulus endotoxin assay by standard methods (3, 4, 7).

**Cell viability.** The results of trypan blue exclusion studies for cells cultured in normal human serum have been presented previously (17). In one experiment with mouse serum, comparable results were obtained (e.g., 20% viability at the end of a 72-h culture period).

**Statistical methods.** The significance of these results was evaluated by using a t test on a Hewlett Packard 981A calculator.

**RESULTS**

**Responses to DNP-BSA.** Representative data on the response of BALB/c thymus cells in 10% normal mouse serum to DNP₃₂₇BSA, as measured by adding [³H]thymidine at the inception of the culture and harvesting at 72 h, are shown in Table 1. This condition was chosen for screening on the basis of previous studies in rabbit bone marrow, which indicated that when radioactive thymidine was added after initiation of the culture the variability in replicate samples was markedly increased. As evaluated by a t test, statistically significant stimulation ($P < 0.05$) was obtained in 10 of 11 experiments (a minimum of five thymuses per experiment) at protein concentrations of 10⁻⁶ M and above. The composite data are given in Fig. 1. Although the stimulation ratios are not large by the usual standards, the buffer background in these and other experiments using DNP-BSA (Fig. 1) ranged from 1,650 to 4,000 counts/min, so a ratio of 1.5 would indicate an antigen-induced increase of 825 to 2,000 counts/min (or 3,300 to 8,000 counts over the 4-min counting period used).

**Responses to con A and other materials.** Responses to BSA and con A are shown in Fig. 1. Significant stimulation to BSA was obtained
TABLE 1. Responses of thymus cells to DNP-BSA

<table>
<thead>
<tr>
<th>Expt</th>
<th>DNP-BSA concn (× 10^{-4} M)</th>
<th>Antigen response(^a)</th>
<th>Buffer control(^b)</th>
<th>Counts/min of [^{3}H]thymidine(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.00</td>
<td>4,341 ± 75.2</td>
<td>3,070 ± 49.7</td>
<td>1,271 (1.41)</td>
</tr>
<tr>
<td></td>
<td>0.33</td>
<td>4,294 ± 151.7</td>
<td></td>
<td>1,224 (1.40)</td>
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<tr>
<td></td>
<td>0.11</td>
<td>3,725 ± 38.2</td>
<td></td>
<td>655 (1.22)</td>
</tr>
<tr>
<td></td>
<td>0.037</td>
<td>3,188 ± 259.01</td>
<td></td>
<td>118 (1.04)</td>
</tr>
<tr>
<td>II</td>
<td>1.00</td>
<td>7,143 ± 35.2</td>
<td>3,993 ± 37.4</td>
<td>3,150 (1.78)</td>
</tr>
<tr>
<td></td>
<td>0.33</td>
<td>6,767 ± 191.8</td>
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<tr>
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<td>0.11</td>
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<td></td>
<td>1,641 (1.41)</td>
</tr>
<tr>
<td></td>
<td>0.037</td>
<td>4,774 ± 165.7</td>
<td></td>
<td>771 (1.19)</td>
</tr>
</tbody>
</table>

\(^a\) Mean of triplicate values counted for 4 min; presented in counts per minute.
\(^b\) Mean of nine or more determinations presented in counts per minute.
\(^c\) Number in parentheses is the ratio of the counts/min of \[^{3}H\]thymidine incorporated into 6% trichloroacetic acid-insoluble material in the presence of 0.04 ml of antigen or mitogen divided by counts/min of \[^{3}H\]thymidine incorporated in the presence of 0.04 ml of PBS.

Fig. 1. Response of normal thymus cells to BSA (O), DNP\(_{6}BSA (\bullet), and con A (\triangle). Cells were cultures at a density of 1.5 \times 10^6 per ml in 10% normal mouse serum. \[^{3}H\]thymidine was added at inception of the culture which was harvested at 72 h. Stimulation ratio was counts per minute for cells in the presence of protein divided by counts per minute for cells in the presence of PBS. Points are the arithmetic mean ± standard error of the mean. Counts per minute for buffer was 3,144 ± 313. Five experiments were conducted for BSA, ten conducted for DNP\(_{6}BSA, and three conducted for con A.

in five out of five experiments at concentrations of 6.4 \times 10^{-5} M and above. Stimulation with BSA was also seen in five out of five experiments in animals injected with PBS 72 h previously (see below). F-ByG was significantly stimulatory in two out of two experiments in un.injected animals and five out of five experiments in PBS-injected animals at concentrations of 2 \times 10^{-5} to 4 \times 10^{-7} M (not shown). In accord with the work of other investigators (2, 23), which appeared while this study was in progress (Lyle et al., Fed. Proc., 31:796, 1972), con A also stimu-

lated a response. The relatively low magnitude of the con A response is discussed below. The threshold of stimulation varied depending on the protein, it was especially different for con A and DNP-BSA with con A stimulating at a 50-fold lower concentration than DNP-BSA. High concentrations of con A resulted in less than maximal stimulation of \[^{3}H\]thymidine incorporation. Additional experiments harvesting and pulsing with \[^{3}H\]thymidine at different times (some of which are presented below) confirmed the ability of DNP-BSA, F-ByG, and con A to stimulate thymidine uptake. In accord with results of previous investigators, we obtained poor responses to phytohemagglutinin (1, 13, 24). In two experiments, we were unable to detect significant responses to polyvinyl pyrrolidone 360 and keyhole limpet hemocyanin.

Possible role of endotoxin in responsiveness to antigen. Since endotoxin has been demonstrated to contaminate commercial animal serum protein preparations (11) and has been reported to stimulate in vitro proliferation of mouse bone marrow cells (19), we considered the possibility that the stimulation by antigen of thymus cells was an effect of endotoxin. None of the preparations induced a pyrogenic response in rabbits (3, 4), but, by the much more sensitive horseshoe crab ameboid cell lysate agglutination technique (7), the preparations could be shown to contain 0.125 to 1 \mu g of endotoxin per mg of protein. The response to DNP-BSA was compared with the response to purified endotoxin (Fig. 2). The endotoxin concentrations used corresponded to the level of endotoxin present as an impurity in antigen-containing tubes. An unequivocal response to DNP-BSA was obtained with the same cells with which endotoxin was nonstimulatory, making it unlikely that contamination by endo-
toxin was responsible for the antigen-induced increase in $[^{3}H]$thymidine incorporation.

**Comprehensive responses in homologous and heterologous sera.** In contrast to the stimulatory effect of DNP-BSA in mouse thymus cells suspended in medium containing isologous serum, no stimulation could be demonstrated in cells in media supplemented with fetal calf, guinea pig, rabbit, or human serum. The response of cells in homologous serum from other mouse strains has not been investigated. Con A stimulation was much greater in media containing human rather than mouse serum, but data in mouse serum are presented for comparative purposes since antigen stimulation could only be obtained in this medium.

**Time course of thymidine uptake.** The time course of thymidine uptake in cells stimulated with foreign proteins and con A was studied. Responses of mouse thymus cells (in human serum) to con A were not demonstrable in the first 10 h ($T_{0}H_{10}$; Fig. 3). Only very marginal responses were demonstrable when $[^{3}H]$thymidine was added at 10 h and the cells harvested at 24 h ($T_{10}H_{24}$). Statistically significant responses to con A were observed in $T_{24}H_{48}$ cultures (stimulation indexes of 2) and in $T_{48}H_{48}$ cultures (stimulation indexes of 4 to 5). The time course of con A-stimulated thymidine uptake in normal mouse serum was similar (not shown).

In contrast to the time course of thymidine uptake in cells stimulated with con A, the response to thymus cells to BSA was relatively rapid (see Fig. 4). Although stimulation indexes were maximal in the $T_{24}H_{48}$ and $T_{48}H_{48}$ experiments, a well-defined and statistically significant response was obtained in a $T_{4}H_{4}$ experiment at all antigen concentrations, and the absolute increase in radioactivity above unstimulated cell backgrounds was actually greater at this time than later in the response. This observation was confirmed by the finding of significant stimulation ($P < 0.05$) on three out of three occasions and two out of two occasions for F-B$_{7}$G and DNP-BSA, respectively, in $T_{4}H_{4}$ experiments (not shown). No response was demonstrable when $[^{3}H]$thymidine was added at the inception of the culture and the cells were harvested at 7 h ($T_{0}H_{7}$; Fig. 4; 2 experiments).

**Responses in cells from antigen-injected animals.** To ascertain the immunological significance of the antigen stimulation, we studied the effect of previous exposure to antigen in vivo on cellular responsiveness. Thymus cells were obtained from groups of 10 animals injected with 2.5 mg of ultracentrifuged BSA and 10 age-matched PBS- or F-B$_{7}$G-injected controls (a total of eight experiments). After 72 h, thymuses were removed from all the animals in each group and processed into suspension culture. Using cells from PBS-injected animals as controls (five experiments), the cells from BSA-injected animals responded significantly less well at all BSA concentrations (Fig. 5B). Responsiveness was decreased at $6 \times 10^{-8}$ M BSA and completely inhibited at $2 \times 10^{-8}$ M and below. Similar results were obtained in three out of three experiments in which animals were pre-injected with BSA or F-B$_{7}$G (e.g., the BSA-injected animals had a significantly decreased

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**Fig. 2.** Response of normal thymus cells to DNP$_3$BSA and endotoxin (one experiment). For culture conditions see Fig. 1. Counts per minute for buffer was 18,860 ± 580. Symbols: ●, DNP$_3$BSA; ○, endotoxin.

**Fig. 3.** Response of mouse thymus cells to con A at various times (three experiments). A, $T_{0}H_{4}$; B, $T_{1}H_{4}$; C, $T_{4}H_{4}$; D, $T_{2}H_{4}$ (T is the time of $[^{3}H]$thymidine addition [hours] and H is the time of culture harvest [hours]).
response to BSA, relative to cells from F-BγG-injected animals; data not shown). Thus, in eight experiments, the preinjection of BSA produced a specific diminution in the in vitro BSA response. Responsiveness to DNP-BSA was also evaluated in cells from BSA-injected and control animals because of the possible importance of the protein carrier in thymus cell recognition of the conjugate. In three individual experiments, the injection of BSA appeared to reduce the DNP-BSA response, but in the group as a whole, the reduction was of borderline statistical significance (Fig. 5A).

**DISCUSSION**

Before the basis for thymocyte responses to antigen can be fully defined, techniques must be developed to allow study of metabolic changes induced by antigen in isolated thymocytes in vitro. The results of this study indicate that selected foreign proteins and hapten-protein conjugates induce DNA synthesis in thymocytes from unimmunized animals in tissue culture. The stimulation was best observed when the cells were incubated in isologous as opposed to heterologous serum, although DNA synthesis in response to con A or alloantigens was more marked in heterologous serum. Incubation in heterologous serum exposed cells to additional foreign antigens raising the background of thymidine uptake (almost fivefold in T₄₃H₁t experiments) and creating the possibility of antigenic competition.

It was necessary to consider the possibility that the stimulation by foreign proteins and hapten-protein conjugates was nonspecific due to contamination with endotoxins or hormones or to the utilization of the proteins for cell nutrition. None of these possibilities seems very likely. (i) A nutritional effect is doubtful because the cultures were carried out in 10% mouse serum, and DNP-BSA and F-BγG were stimulatory at 10⁻⁶ M concentrations. Moreover, the cells were cultured at a low density and they replicated at a relatively slow rate, making it unlikely that serum proteins would be depleted under the usual culture conditions. (ii) It is also improbable that endotoxin contamination of the proteins is involved since purified endotoxins could not be shown to stimulate DNA synthesis. (iii) The possibility of hormonal contamination is raised by the studies of McManus and Whitfield (18) on hormonal stimulation of rat thymocytes in tissue culture. They demonstrated that, in media containing limiting amounts of calcium, a variety of hor-

**FIG. 4. Response of mouse thymus cells to BSA at various times (two experiments).** Cells were cultured in 10% normal mouse serum. A, T₄₃H₁t; B, T₄₃H₁u; C, T₁₇H₁t, and D, T₄₃H₁u.

**FIG. 5. DNA synthetic responses of thymus cells from mice injected intraperitoneally either with PBS (●) or 2.5 μg of centrifuged B (O) 72 h previously.** Cells (1.5 × 10⁶ per ml) were cultured in 10% normal mouse serum. [³H]thymidine was added at the inception of the culture. Cells were harvested after 72 h (T₄₃H₁t). Thymocytes from a total of 20 animals were used in each experiment, pooling cells from 10 BSA-injected and the 10 PBS-injected mice. A, Cells stimulated in vitro with DNP-BSA. B, Cells stimulated in vitro with BSA. The results are presented as the mean (± standard error of the mean) stimulation ratio for five experiments. At each of the BSA concentrations, the response in the cells from BSA-injected animals is statistically less than the response in PBS-injected animals (P < 0.05, Student t test). Evaluating the experiments individually in five of five experiments, the response at the two intermediate concentrations of BSA was significantly reduced in cells from BSA-injected animals. The differences in the mean stimulation ratios with DNP-BSA are of borderline statistical significance.
monal agents which raise thymocyte cyclic adenosine 5'-monophosphate levels or promote the entry of calcium into cells stimulate DNA synthesis, the effect occurring within the first 6 h (18). Since we failed to observe an antigen-induced increase in DNA synthesis in T₄H₄ cultures, and since adequate amounts of calcium were present in the medium and stimulation was obtained with highly purified, extensively dialyzed proteins, hormonal contamination does not appear to explain the stimulatory action of the above proteins in our system.

It is unlikely that a small number of contaminating B cells or mature peripheral blood T cells in the thymocyte preparations could account for the responses seen: (i) we have been unable to stimulate mouse bone marrow cells with antigens under similar conditions; (ii) the cultured thymocytes failed to respond to endotoxin as might have expected if functional B cells had been present; (iii) the responses to antigen in thymocytes were markedly inhibited by antitheta antibody (not shown); and (iv) as discussed below, the kinetics of antigen stimulation differ in thymocytes and peripheral lymphocytes.

A better argument that the in vitro stimulation of mouse thymus cells by foreign macromolecules is immunologically mediated comes from antigen injection studies. In cells from animals injected with ultracentrifuged BSA 72 h previously, the response to BSA was decreased or absent. Our findings in mouse thymocytes from antigen-injected and control animals are reminiscent of those of Singhal and Richter (22) in rabbit bone marrow cells. They found that rabbit marrow cells take up increased amounts of [³H]thymidine in response to foreign proteins in vivo. The response to a particular protein was specifically abolished in animals given large amounts of the same protein intravenously several days previously. Our own observations with rabbit bone marrow (Eisen et al., Fed. Proc., 30:649, 1971), as well as those of Mond and Thorbecke with both rabbit and mouse bone marrow (20), confirm the increase in DNA synthesis in response to foreign proteins, but fail to confirm that the response is ablated in preinjected animals. Because of the lack of an effect of prior immunologic exposure, Mond and Thorbecke have concluded that the bone marrow response to foreign proteins in these two species is nonspecific. Although this interpretation may well be correct, the cell transfer studies of Chiller et al. (6) indicate that it is relatively difficult to induce tolerance in mouse bone marrow cells in vivo; more extensive studies are needed. Irrespective

of the outcome of further experiments with bone marrow cells, since BSA responsiveness is altered in thymus cells from BSA-injected animals, the probability is that the thymocyte response is immunologically specific. Chiller et al. have previously demonstrated that the ability of thymocytes to specifically act synergistically with normal bone marrow cells when transferred to irradiated animals can be diminished by preinjection with large quantities of a given antigen (6), and it therefore seems likely that the change in the in vitro response observed in this study is analogous to high antigen dose tolerance.

One of the most interesting observations was in regard to the time course of thymidine uptake in thymus cells responding to foreign proteins. The absolute increase in thymidine uptake was greater from 6 to 24 h than it was at a later time, indicating a surprisingly early DNA synthetic response to antigen in vitro. An early (within 24 h) T-cell proliferative response in mouse spleen cells exposed to foreign erythrocytes and haptenated erythrocytes in vivo has been inferred on the basis of measurements of the immunoglobulin M plaque-forming cell response before and after lethal irradiation (R. Bachvaroff and F. T. Rapaport, Fed. Proc., 32:1021, 1973). In mice, as well as in other species, the response of peripheral lymphoid cells to antigen is very different in that the time of maximal DNA synthesis is delayed for at least 72 h. In contrast to the early thymocyte response to foreign proteins, the maximal DNA synthetic response to con A occurs at about the expected time, well beyond the period of maximal foreign protein stimulation, suggesting that foreign proteins and con A stimulate DNA synthesis by different mechanisms. Further studies are needed to define whether the unexpectedly rapid increase in DNA synthesis in thymocytes exposed to foreign protein is because the cells are at a different stage of development than peripheral thymus dependent cells or because the response involves a special class of lymphocytes contained early only in the thymus.

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LITERATURE CITED


