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Cells Involved in the Immune Response

XXII. THE DEMONSTRATION OF THYMUS-SPECIFIC ANTIGENS IN THE RABBIT

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Summary. Horse anti-rabbit thymus cell serum (HARTS) was obtained by immunizing a horse with rabbit thymocytes intravenously at weekly intervals for 3 weeks. The horse was bled 2 weeks later and the antiserum was analysed for its cytotoxic activity with respect to the lymphocytes of the various lymphoid organs. It was demonstrated that the cytotoxic activity of the antiserum was several orders of magnitude greater for thymus cells than for cells of the other organs tested. Only thymus and lymph node cells were capable of absorbing the thymocytotoxic activity of the antiserum; however, ten to fifteen times as many lymph node cells as thymus cells were required to neutralize the thymocytotoxic activity of the serum. Absorption of the antiserum with the cells of the other lymphoid organs (spleen, bone marrow, appendix, sacculus rotundus, Peyer's patches and circulating leucocytes) resulted in a slight but significant decrease in the thymocytotoxic activity. At no time was the thymocytotoxic activity completely absorbed with cells of these organs. The cytotoxic activity of the antiserum with respect to the cells of the different lymphoid organs other than the thymus could be abolished following absorption of the antiserum with the cells of any of the lymphoid organs. On the basis of our data, it is concluded that (a) the thymocytes possess two groups of antigens—one thymocyte specific and one common to all rabbit lymphocytes and (b) only the lymph nodes of all the lymphoid organs other than the thymus possess significant numbers of thymus-derived or T-cells. However, the proportion of these cells in the lymph node does not exceed 7–10 per cent, a figure much lower than that found in the lymph nodes of the mouse. Less than 1 per cent of the circulating lymphocytes in the rabbit are T-cells.

INTRODUCTION

Within the past few years, it has been unequivocally demonstrated that the thymus cells in the mouse possess antigens unique to the thymus, not represented on lymphoid cells present in the other lymphoid organs with the exception of the thymus-derived lymphocytes (Raff, 1969; Raff and Wortis, 1970; Raff, 1971; Schlesinger and Yron, 1969, 1970). Similar results have been obtained in the rat (Colley, Malakian and Waksman, 1970; Waksman, 1971; Bachvaroff, Galdiero and Grabar, 1969; Potworowski and Nairn, 1967)

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and in the chicken (McArthur, Chapman and Thorbecke, 1971). More recently, it has been demonstrated that mouse bone marrow lymphocytes also display antigenic specificity (Raff, 1971a; Raff, Nase and Mitchison, 1971; Niederhuber, 1971), thus providing two markers with which to distinguish lymphoid cells in the various lymphoid organs.

Investigations in this laboratory have centred about the organ localization of the functionally different lymphoid cells in the rabbit. The antigen reactive cell (ARC) has been localized essentially to the bone marrow (Abdou and Richter, 1969; Abdou and Richter, 1970; Richter, Rose and Abdou, 1970). The antibody-forming cell (AFC), on the other hand, appears not to be localized to any specific lymphoid organ but to be distributed in all the lymphoid organs (Richter and Haasz, 1972). However, it is probable that, ontogenetically, the precursor of the AFC cell arises in one or several of the lymphoid organs and then infiltrates the remaining lymphoid organs. The acquisition of antisera specific in their reactivity toward cells present in or derived from a particular lymphoid organ would greatly facilitate the resolution of the problem—that is, the original organ of origin of the AFC. To accomplish this end, we have prepared antisera directed toward rabbit thymus, bone marrow, appendix, sacculus rotundus and spleen lymphoid cells. The objective of this investigation is to demonstrate the presence of thymus-specific antigens in the rabbit.

METHODS AND MATERIALS

The protocol followed in this investigation is diagrammatically presented in Fig. 1.

Preparation of cells

Adult New Zealand White rabbits were used as lymphoid cell donors. They were killed by the intravenous injection of Nembutal (50 mg/kg body weight) and the spleen, thymus, appendix, sacculus rotundus, Peyer's patches, popliteal lymph nodes and bone marrow were removed without delay. The manner of preparing cell suspensions from these organs has been described in detail elsewhere (Abdou and Richter, 1969; Singhal and Richter, 1968). Briefly, the organs were collected in medium 199 (Microbiological Associates, Bethesda, Md, U.S.A.) and cut into small fragments. The fragments were then placed onto a wire mesh (50 mesh) and the cells were expressed following gentle pressure on the fragments. The cells were collected in medium 199 containing 5 per cent normal rabbit serum (Microbiological Associates), centrifuged at 1000 rev/min for 10 minutes and resuspended in the medium to the desired cell concentration. Circulating lymphocytes were obtained by bleeding directly from the heart into a syringe containing a saline solution of 6 per cent dextran (mol. wt 200,000 –250,000) and heparin to give a final ratio of blood to dextran of 2:1 and a final concentration of 50 units heparin per ml of blood collected (Daguillard and Richter, 1969). The blood was transferred into 15-ml plastic tubes and allowed to sediment at 37°. The buffy coat was then pipetted off and the cells were suspended in medium 199. They were centrifuged at 2,000 rev/min for 10 minutes and resuspended in medium 199 to the desired cell concentration.

Cell suspensions prepared from the kidney, brain and liver of normal rabbits were prepared in the manner described above for the cells of the lymphoid organs. Red blood cells were obtained following the separation of the buffy coat containing the white cells. The red cells were suspended in medium 199, centrifuged at 2,000 rev/min for 10 minutes and resuspended in medium 199.

Preparation of horse anti-rabbit thymocyte serum (HARTS)

Horse anti-rabbit thymocyte serum was prepared in the following manner: a cell suspension (500×10^8 cells) of rabbit thymus was prepared and injected intravenously into normal, adult horses three times at 7-day intervals. The animals were bled 2, 3 and 5 weeks after the last injection of the antigen and the antiserum was obtained by centrifugation of the clotted blood. The sera were stored at $-10°$ until used.

Absorption of the horse anti-rabbit thymocyte serum (HARTS)

The cell suspensions (usually 1 ml containing approximately 10^6–5×10^8 cells) prepared from the various lymphoid organs were centrifuged at 1000–1500 rev/min for 10 minutes. The supernatants were decanted and 1-ml aliquots of the antiserum (in the appropriate dilution) were added to the tubes. The cells were then resuspended in the antiserum and the tubes were incubated for 30 minutes at room temperature with occasional shaking. The tubes were then centrifuged at 3000 rev/min for 10 minutes and the supernatants (absorbed antiserum specimen) were stored at 4° or $-10°$ until used.

Analysis for cytotoxicity

The test used to determine the lymphocytotoxic properties of the anti-thymus serum is essentially that of Colley, Malakian and Waksman (1970) and Motta (1968). The lymphoid cells were suspended in medium 199 to a cell concentration of $2{\cdot}5 \times 10^6$ per ml. One-tenth millilitre of this cell suspension was then placed in a test tube to which was added 0·1 ml of the test antiserum and 0·1 ml of complement (C′). The antiserum was decomplemented (30 minutes, 56°) prior to use. The source of the C′ was fresh, frozen guinea-pig serum diluted three-fold to five-fold. Control tubes consisted of normal horse serum in place of the suspected antiserum, C′ alone or antiserum alone. The tubes were incubated in a 37° water bath for 60 minutes following which they were immersed in an ice bath. One-tenth millilitre of a trypan blue solution (prepared by mixing 4 volumes of a 0·2 per cent solution of trypan blue with 1 volume of a 4·25 per cent solution of sodium chloride) was added to each tube and the cells were analysed by light microscopy using a Neubauer Chamber. The proportion of stained (dead) or unstained (live) cells was determined on the basis of the analysis of approximately 250–300 cells. The cytotoxicity index of the antiserum was calculated as follows:

$$\text{Cytotoxicity index} = \frac{\text{DE–DC}}{\text{100–DC}} \times 100$$

where DE is the percentage of dead cells in the tube containing the antiserum with C′ and DC is the percentage of dead cells in the control tube containing normal horse serum and C′.

RESULTS

Each of the experiments reported upon below was repeated a minimum of four times. With few exceptions, the results were consistently reproducible. Therefore, to facilitate presentation of the data, only results of representative experiments, rather than results of all the experiments in each series, are given in the figures.

As can be seen in Fig. 2, the cytotoxic titre of the HARTS was much higher with respect to rabbit thymus cells than with the cells of any of the other lymphoid organs

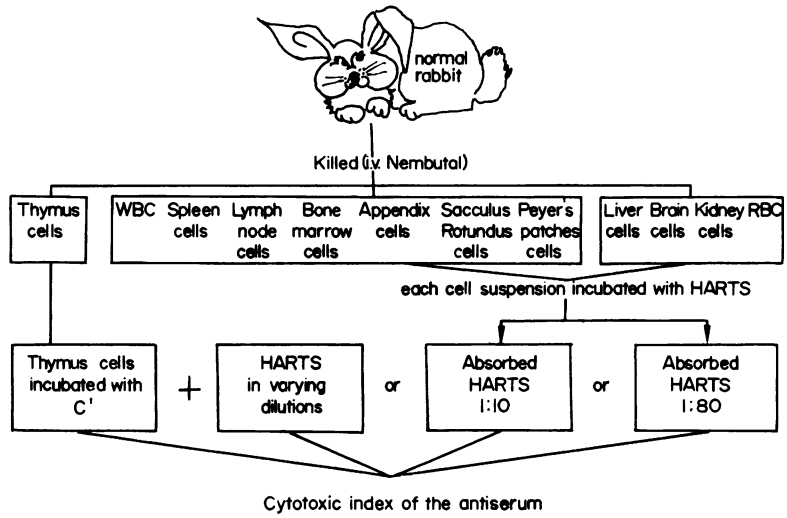

FIG. 1. Protocol for the demonstration of thymus-specific antigens in the normal adult rabbit. (HARTS = horse anti-rabbit thymus cell serum).

tested. The lymphocytotoxic titres (100 and 50 per cent cell lysis) of the HARTS were about the same for all the cells tested other than the thymus cells. The dilutions of the HARTS capable of effecting 90–100 per cent and 50 per cent lysis of thymus cell were

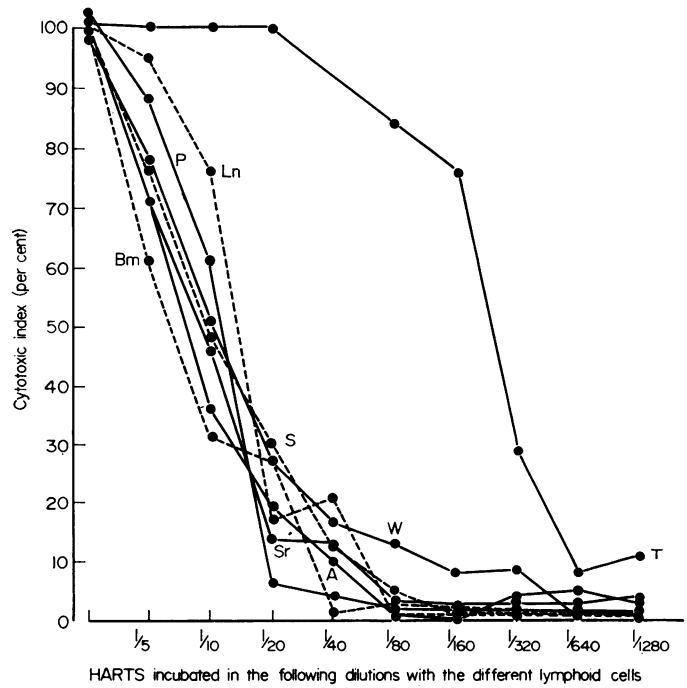

FIG. 2. The lymphocytotoxic activity of horse anti-rabbit thymus cell serum with respect to the lymphoid cells of the different lymphoid organs. T = thymus cells. A = appendix cells; S = spleen cells; P = Peyer's patches cells; Ln = lymph nodes cells; Sr = Sacculus rotundus cells; W = leucocytes; Bm = bone marrow cells.

10–100 times higher than those required to effect similar degrees of lysis of the cells of any of the other lymphoid organs.

Absorption of the antiserum with kidney, liver, brain or red blood cells did not significantly alter its cytotoxic properties with respect to thymus cells (Fig. 3).

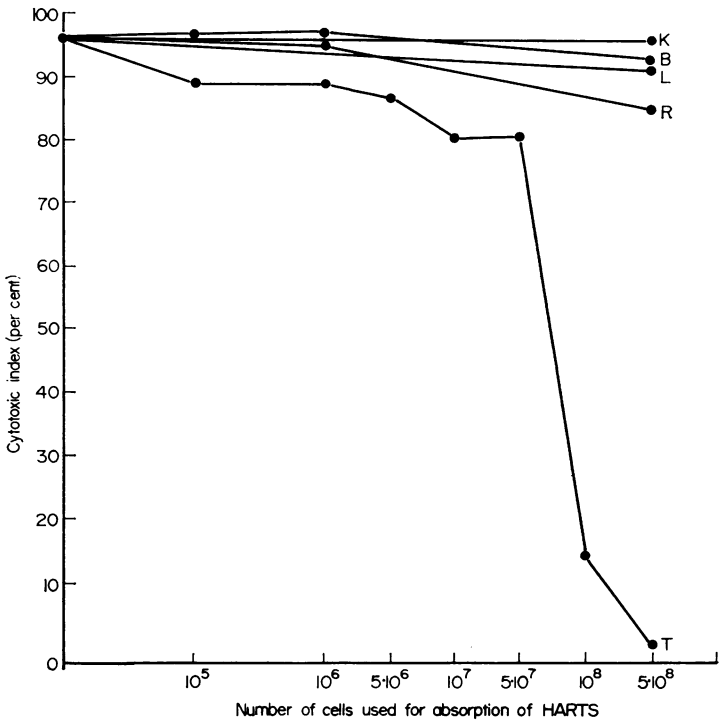

FIG. 3. The lymphocytotoxic activity of horse anti-rabbit thymus cell serum (diluted ten-fold) following absorption with rabbit thymus, brain, kidney, liver and red blood cells. Cytotoxicity of absorbed HARTS with respect to rabbit thymus cells. T = thymus cells; K = kidney cells; B = brain cells; L = liver cells; R = red blood cells.

The cytotoxic activity of the HARTS (diluted ten-fold) absorbed with the different lymphoid cell preparations is presented in Fig. 4. Only cells of the popliteal lymph nodes were able to absorb appreciable cytotoxic activity with respect to the thymus cells as target cells. Absorption of the HARTS (1:10) with 5×10^8 lymph node cells depressed the cytotoxic index from 80 per cent to 50–60 per cent. However, absorption of the HARTS (1:10) with as few as 10^7–10^8 thymus cells resulted in complete loss of cytotoxic activity. Absorption of HARTS (1:10) with as many as 5×10^8–10^9 cells of any of the other lymphoid organs did not result in any significant loss of cytotoxic activity with respect to the target thymus cells.

Somewhat different but supporting results with the HARTS diluted eighty-fold are presented in Fig. 5. Invariably, absorption of the HARTS (1:80) with 5×10^7–10^8 lymph node lymphocytes resulted in a complete or an almost complete loss of cytotoxic activity, comparable to the effect observed following absorption of the HARTS (1:80) with 5×10^6–10^7 thymus cells. The cytotoxic activity of the HARTS (1:80) absorbed with any of the other lymphoid cells was also significantly diminished compared to that of the unabsorbed control but at no time did the cytotoxic activity of these absorbed HARTS (1:80) ap-

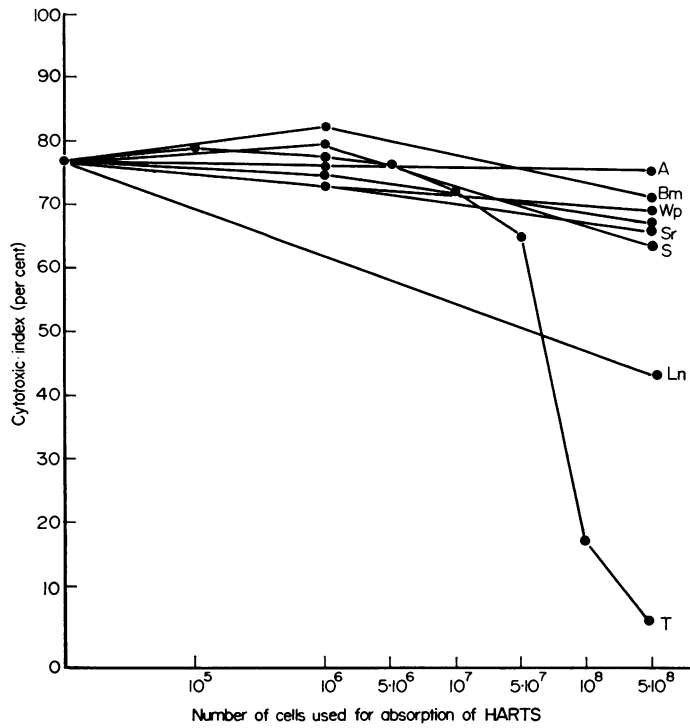

FIG. 4. The lymphocytotoxic activity of horse anti-rabbit thymus cell serum (diluted ten-fold) following absorption with varied numbers of cells of the different lymphoid organs. Cytotoxicity of absorbed HARTS with respect to rabbit thymus cells. T = thymus cells. A = appendix cells; P = Peyer's patches cells; Ln = lymph node cells; Sr = Sacculus rotundus cells; W = leucocytes; S = spleen, Bm = bone marrow cells.

proach or become asymptotic with the X-axis. However, circulating lymphocytes were invariably more efficient in their absorptive capacity than the cells of the spleen, appendix, sacculus rotundus or Peyer's patches (Fig. 5).

The cytotoxic activity displayed by the HARTS with respect to the cells of the lymphoid organs other than the thymus could be absorbed with cells of any of the lymphoid organs. For example, the cytotoxicity of HARTS with respect to spleen cells or lymph node cells could be absorbed with cells of any of the lymphoid organs. Furthermore, the numbers of lymphoid cells of the different organs required to completely absorb this cytotoxic activity were almost equal in number. This cytotoxic activity towards non-thymic lymphocytes could also be absorbed with kidney, liver and brain cells, although 10–20 times as many of these cells were required.

DISCUSSION

The long-range objective sought in this investigation is the acquisition of antisera directed specifically toward parenchymal cells of the anatomically-distinct lymphoid organs of the normal rabbit—the thymus, bone marrow, spleen and the gut-associated lymphoid organs (appendix, Peyer's patches and sacculus rotundus). The availability of such organ-specific antisera might permit the identification of the organ source(s) of the uncommitted, virgin antibody–forming cell (AFC) or its precursor. Although the antibody-synthesizing

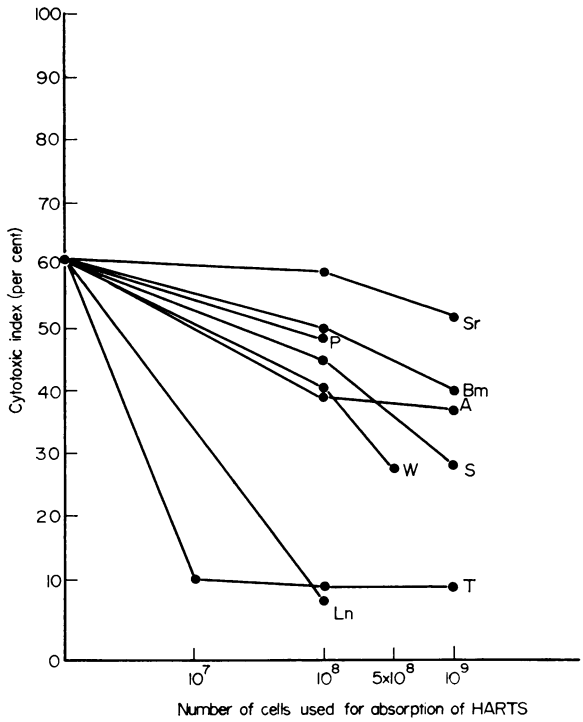

FIG. 5. The lymphocytotoxic activity of horse anti-rabbit thymus cell serum (diluted eighty-fold) following absorption with varied numbers of cells of the different lymphoid organs. Cytotoxicity of absorbed HARTS with respect to rabbit thymus cells. T = thymus cells; S = spleen cells. A = appendix cells; P = Peyer's patches cells; Ln = lymph node cells; Sr = Sacculus retundus cells; W = leucocytes; Bm = bone marrow cells.

AFC is initially detected in the spleen of the rabbit following immunization (Abdou and Richter, 1969), it does not necessarily follow that this cell originates from a precursor within the spleen. Indeed, the AFC or its precursor may be the progeny of lymphoid cells which are normally localized to another lymphoid organ from which these cells are released into the circulation following antigenic stimulation. The spleen may simply provide the most fertile grounds for the further maturation of these cells to overt AFCs.

The immediate objective of this investigation was to demonstrate the antigenic specificity of rabbit thymus cells or, conversely, to obtain an antiserum specific for rabbit thymus cells. This objective was accomplished by absorbing the horse antiserum to rabbit thymocytes (HARTS) with cells of the other lymphoid organs. Thus, the lymphocytotoxicity exhibited by the HARTS toward the cells of the various lymphoid organs other than the thymus—spleen, bone marow, lymph node, appendix, sacculus rotundus and Peyer's patches cells and the circulating lymphocytes—could be completely neutralized following absorption of the antiserum with cells of any of these organs. However, the absorbed HARTS still displayed the capacity to lyse 80–90 per cent of thymus cells it was incubated with. Thus, the antibodies directed specifically to the thymus cells could not be absorbed by cells of any of the other lymphoid organs. These data suggest that the rabbit thymus cell possesses at least two antigens or groups of antigens, one distinct for the thymus cell

and one which is common to lymphocytes in general, irrespective of their source. A similar conclusion was arrived at by Colley *et al.* (1970) with respect to the antigens present on the lymphoid cells in the rat. Furthermore, it was also observed that the cytotoxic activity of HARTS toward lymphocytes other than thymocytes could be absorbed with brain, kidney and liver cells, although these cells were far less efficient in their absorptive capacity than were cells of the different lymphoid organs. It would therefore appear that, as in the rat (Colley *et al.*, 1970) an antigen or antigens common to all lymphocytes are also present in kidney, liver and brain cells, although to a much lesser degree.

The results may be compared to those obtained for lymphoid cells in the mouse (Raff, 1969; Raff and Wortis, 1970; Raff, 1971; Schlesinger and Yron, 1969, 1970) and in the rat (Colley, Malakian and Waksman, 1970; Waksman, 1971; Bachvaroff, Galdiero and Graber, 1969; Potworowski and Nairn, 1967) in which it was demonstrated that thymus cells as well as thymic-derived cells in the peripheral lymphoid organs are characterized by the presence of a specific surface antigen, referred to as the θ-antigen. Thus, it has been demonstrated that thymus-derived or T-cells can be detected in the mouse lymph node and spleen by virtue of their susceptibility to lysis following contact with C′ and anti-θ serum produced in a different strain of mouse. It has also been demonstrated that, in the mouse, the brain possesses the θ-antigen(s) (Greaves and Raff, 1971; Raff, 1969) since the thymocytotoxic activity of the antiserum can be absorbed by incubation with brain tissue. However, such a situation does not appear to exist in the rabbit since the cytotoxic activity of the anti-thymus cell antiserum toward thymus cells was not affected by absorption of the antiserum with brain tissue. Nevertheless, the results do not rule out the possibility that the rabbit brain may possess one or more of the thymus-specific antigens since absorption of the anti-thymus serum with brain tissue would still leave antibodies specific in their reactivity to thymus cells.

It is noteworthy that only the lymphocytes of the popliteal lymph nodes were able to absorb completely the anti-thymus cell activity of the diluted (1:80) HARTS. Absorption of HARTS with cells of any of the other lymphoid organs with the exception of the thymus resulted in a reduction of the thymocytotoxic activity of the antiserum. The cytotoxic index of such absorbed HARTS was rarely decreased to below 40 per cent. Since approximately 10–15 times as many lymph node cells as thymus cells were required in order to absorb the thymocytotoxic activity of the HARTS, it may be argued that about 7–10 per cent of the lymph node lymphocytes are T-cells or cells which carry the thymus-specific antigen(s). None of the other lymphoid organs appear to normally possess T-cells since cells of these organs were incapable, irrespective of the number used, to absorb the thymocytotoxic activity of the antiserum. Thus, unlike the situation which exists in the mouse, it would appear that in the rabbit only the lymph nodes normally contain T-cells.

Criticism which may be voiced with respect to the current investigation in the rabbit may arise from the fact that heterologous (or xenogeneic) antiserum rather than homologous (or allogeneic) antiserum was used, unlike investigations conducted in the mouse in which mice of one strain have been used to produce antisera to thymocytes of mice of a different strain. However, the use of heterologous anti-rabbit thymocyte serum is a necessary condition imposed on the investigator in view of the fact that only outbred rabbits are available for investigation of this type. It might have been anticipated that a third group of antigens would be detected in the thymus cells—organ-specific, non-species-specific antigens. However, such does not appear to be the case since HARTS is not at all cytotoxic with respect to human thymus cells, nor is rabbit anti-human thymocyte serum cytotoxic

with respect to rabbit thymocytes (de la Noue and Richter, unpublished results). It is also evident, from the results of the absorption experiments, that the antibodies in HARTS are not directed toward transplantation or histocompatibility (H) antigens. If such were the case, then absorption of the HARTS with the lymphocytes of any of the lymphoid organs should have abolished the thymocytotoxic activity of the antiserum, since all of these cells possess H antigens.

Implicit in the interpretation of the results presented here and those of other investigations in the mouse and the rat is the assumption that the number of thymus-specific antigens present on each T-cell following peripheralization is identical to the number of these antigens present on the surface of the thymocyte. This may not be the case, however. It has been stated that the thymus cells in the mouse lose θ-antigens (Raff, 1971; Aoki, Hammerling, de Harven, Boyse and Old, 1969) and TL antigens (Boyse, Old, Stockert and Shigeno, 1968; Raff, 1971; Raff and Cantor, 1971) following peripheralization. However, Raff (1971b) has recently demonstrated that there exists in the mouse thymus a subpopulation of TL negative cells which appear to resemble peripheralized T-cells. It is therefore probable that the mouse thymocyte loses the TL antigens and some of the θ-antigens and gains histocompatibility antigens prior to emigration from the thymus to the peripheral lymphoid organs. If these arguments with respect to mouse T-cells were to be extrapolated to rabbit cells, the number of T-cells in the spleen and lymph nodes would be greater than that calculated by extrapolation from absorption experiments, since the basis for the calculation of the number of T-cells in the peripheral lymphoid organs is that the thymus-specific antigens are present in equal numbers on the thymocyte and peripheralized T-cell. Such a condition would dictate that the cells be less sensitive to the cytotoxic activity of the anti-thymus cell serum at threshold cytotoxic concentration than the thymocytes themselves, as has been shown with mouse T-cells (Raff, 1971). However, one cannot ascertain on the basis of our results whether this condition can be considered to exist for rabbit T-cells owing to the probability that the anti-rabbit thymocyte serum, having been prepared in the horse, is directed toward all of the thymus-specific and thymus-non-specific antigens rather than toward any one, well-defined antigen. The determination whether the rabbit thymocytes lose thymus-specific antigens following their migration to the peripheral lymphoid organs or whether the peripheral T-cells represent a subpopulation of thymus cells must await the acquisition of a more specific antiserum directed toward select antigens on the surface of the rabbit thymocyte.

The results suggest that the relationships which exist between the thymus and the peripheral lymphoid organs, particularly with respect to the relative number of T-cells in the spleen and lymph nodes, are different in the three animal species analysed thus far—the mouse, the rat and the rabbit. In the adult mouse, it has been stated that as many as 70 per cent of the circulating lymphocytes, 35–45 per cent of the spleen cells and 60–75 per cent of the lymph nodes cells are T-cells (Raff, 1971; Greaves and Raff, 1971; Parrott and de Sousa, 1971; Raff and Owen, 1971). In the adult rat, fewer than 7 per cent of the lymph nodes cells, spleen cells and circulating lymphocytes appear to be T-cells (Colley, Malakian and Waksman, 1970). On the basis of the results reported here, about 7–10 per cent of the cells in the lymph nodes and less than 1 per cent of the circulating lymphocytes in the adult rabbit appear to be T-cells while the spleen appears to be totally devoid of these cells. The significance of these findings with respect to the role which the different lymphoid organs play in the immune response in the immunologically mature rabbit is currently under investigation.

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