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The interaction of lymphocytes with autologous red cells

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There is a relatively large degree of adherence of rabbit thymocytes to autologous or homologous mature and nucleated red cells, but not to heterologous red cells. The adherence is augmented by sera and a variety of protein solutions. It is inhibited by 4×10^{-5} M iodoacetate or pretreatment of red cells by 0.01 to 0.5 per cent trypsin solution. Thymocytes obtained from one- to 7-day-old rabbits adhere to rabbit red cells to a larger extent than do thymocytes of adult rabbits. Significant autologous or homologous thymocyte-red cell adherence also occurred in the rat but not in the mouse or guinea pig. Heterologous adherence was not evident except in mixtures containing guinea pig thymocytes and rabbit red cells or mouse thymocytes and rat red cells. It is postulated that the adherence of thymocytes to the red cells occurs through nonimmune mechanisms and that it is likely to affect the homing patterns of the lymphocytes.

The adherence of lymphocytes to cells has been associated with several characteristic responses of the lymphocytes. Thus, lymphocytes may adhere to and destroy target cells during delayed-type immune responses in vitro.¹ Lymphocytes likewise adhere to macrophages in mixed leukocyte cultures and blast transformation of the lymphocytes.² Recent studies have indicated that thymocytes adhere to a variety of autologous cell types. Thus, guinea pig thymocytes adhere to autologous macrophages and to a lesser extent to autologous polymorphonuclear leukocytes.^{3, 4} Rat thymocytes adhere to autologous mast cells, and rabbit thymocytes adhere to autologous red cells.⁵ The following study will describe in detail the adherence of lymphocytes to autologous red cells.

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MATERIALS AND METHODS

Animals

Animals used in this study consisted mainly of 2- to 6-month-old and 1- to 7-day-old New Zealand white rabbits. The latter have been referred to as newborn rabbits. Other animals used were 30 to 40 grams Swiss Webster mice, 75 to 200 grams Sprague-Dawley rats, and 200 to 450 grams Hartley guinea pigs. All animals were randomly bred and not distinguished on the basis of sex.

Salt and protein solutions

Unless otherwise specified, cells and reagents were prepared in a phosphate-buffered modified Hanks solution.⁶ Fetal bovine serum was obtained from Microbiological Associates (Bethesda, Md.). All other sera were obtained from cardiac blood of animals and stored at -25 to -30° C. Proteins utilized were rabbit albumin fraction V powder (Pentex), rabbit gamma globulin fraction II (Pentex), and purified pigskin gelatin (Eastman Organic Chemicals). Lyophilized 200 U. per milligram trypsin (Worthington) was employed.

Cells

White cells were obtained according to methods described previously.⁴ Rabbit lymph node lymphocyte suspensions contained 84 to 97 per cent small lymphocytes and 3 to 16 per cent larger cell types. Rabbit thymocyte suspensions were composed of 95 to 98 per cent small lymphocytes and 2 to 5 per cent larger cells. There were usually only traces of red cells in the lymphocyte or thymocyte preparations. Bone marrow and spleen cell suspensions contained heterogeneous cell populations. Among the cell types present in suspensions of bone marrow cells obtained from adult and newborn rabbits and in suspensions of spleen cells obtained from newborn rabbits were mature and nucleated red cells (erythroblasts). The latter cells were identified through morphologic criteria described by Bessis⁷ and by the presence of hemoglobin-like staining material in many of the cells in Wright's-stained formaldehyde vapor-fixed smears.

Rabbit, guinea pig, rat, and mouse red cells were obtained by cardiac puncture. Sheep and chicken red cells in Alsever's solution were obtained from GIBCO (Madison, Wis.). All cells were washed 3 times with modified Hanks solution, stored in the salt solution at about 4° C., and usually utilized during the day of preparation.

Standard cell mixtures

Lymphocyte suspensions were mixed with red cell suspensions to form mixtures which contained the following: (1) 0.1 ml. of 1.6×10^7 lymphocytes per milliliter, (2) 0.1 ml. of one per cent (by volume) of red cells, and (3) 0.1 ml. of modified Hanks solution with or without the reagent tested. The mixtures either were incubated at 37° C. for 60 minutes without sedimentation or were sedimented without prior incubation. Sedimentation of the cell suspensions was achieved by centrifugation at 1,700 to 2,000 r.p.m. for 15 to 30 seconds. The resulting sediment was resuspended by a pipette and a drop transferred to a slide for observation under a phase microscope.

Evaluation of adherence

The degree of adherence of small lymphocytes to red cells was measured by the percentage of 100 randomly chosen small lymphocytes which adhered to one or more red cells. Gross differences in the degree of adherence were noted by comparing the number of red cell-lymphocyte aggregates in 100 low power (10× objective) microscopic fields. This was useful in comparing the degree of adherence in mixtures which contained dense red cell-lymphocyte aggregates.

Pretreatment of cells

Nine-tenths milliliter of 1.6×10^7 lymphocytes per milliliter or 0.9 ml. of 1 per cent red cells were incubated for 60 minutes at 37° C. with 0.1 ml. of twofold dilutions of the

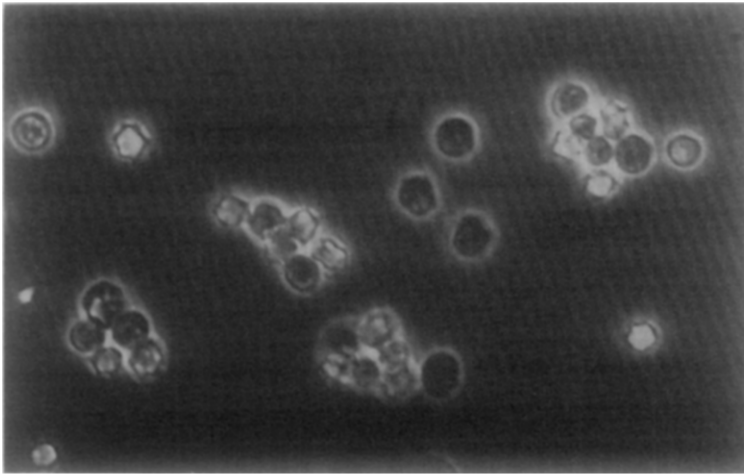


FIG. 1. Rabbit thymocytes and autologous red cells in 100 per cent modified Hanks solution. Suspensions were sedimented immediately after preparation and observed under the phase microscope.

reagent tested. The reagent was then removed, the cells washed 3 times, and resuspended at their original concentrations in modified Hanks solution. Adherence activity of the pretreated cells was tested by the standard procedures described above.

RESULTS

Adherence in the absence of serum

Thymocyte-red cell aggregates containing 1 to 4 thymocytes and 1 to 4 red cells formed in mixtures containing rabbit thymocytes and autologous red cells (Fig. 1). Aggregates containing a larger number of red cells and thymocytes were occasionally evident. Significant adherence in the absence of serum occurred immediately after thymocytes were mixed with autologous red cells and sedimented for 15 to 30 seconds at room temperature. Additional incubation of the mixtures for 60 minutes at 37° C. did not significantly increase the number of thymocyte-red cell aggregates. There were few or no aggregates in thymocyte suspensions to which red cells were not added or in red cell suspensions to which thymocytes were not added.

The degree of lymphocyte-red cell adherence, as measured by the percentage of lymphocytes which adhered to the red cells, is illustrated in Fig. 2, Column 1. In contrast to thymocytes, there was relatively little adherence of lymphocytes obtained from most lymph nodes to autologous red cells (Fig. 2, Column 3). The adherence of rabbit thymocytes to homologous red cells was not significantly different from their adherence to autologous red cells. There was little or no adherence of rabbit thymocytes to guinea pig, rat, mouse, dog, sheep, and human red cells.

Adherence in the presence of serum

Autologous serum up to a dilution of about 1/100 augmented thymocyte-red cell adherence. This was evident by the facts that a larger proportion of thymo-

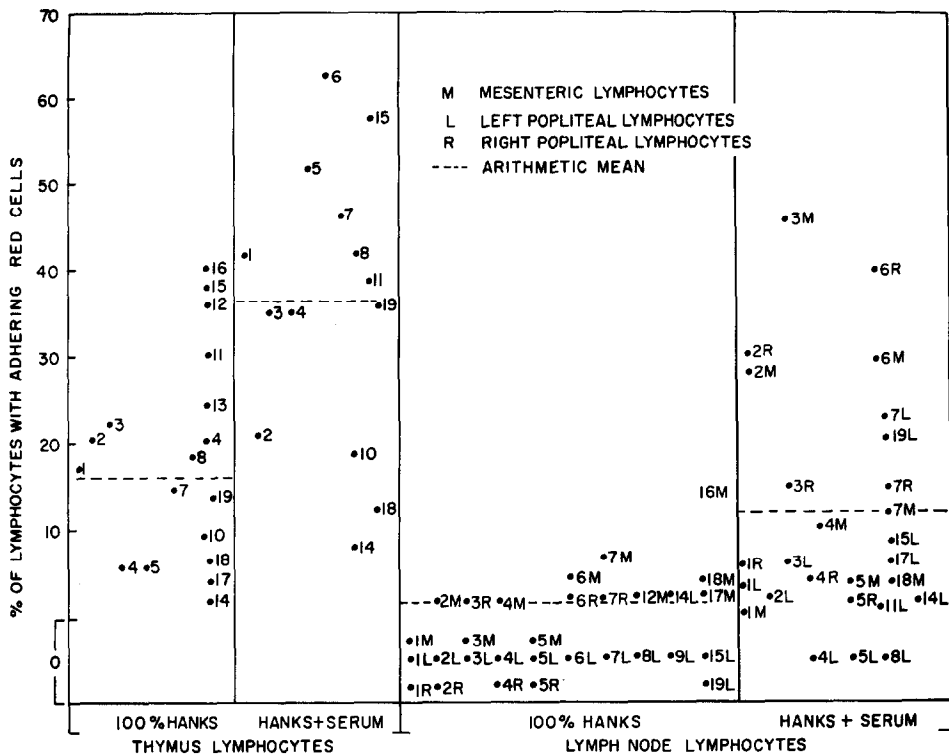


FIG. 2. Lymphocyte-red cell adherence in mixtures containing lymphocytes from 19 different rabbits in 19 consecutive experiments. Numbers inside the chart signify the individual rabbits from which lymphocytes were obtained. **Hanks + Serum** tubes contained autologous serum at 1:4 dilution. All mixtures were observed after incubation at 37° C. for 60 minutes.

cytes adhered to red cells (Fig. 2, Column 2) and by the formation of larger thymocyte-red cell aggregates. An additional effect of serum was seen by an increase in the number of thymocytes which adhered to red cells through the concave areas of the red cells (Fig. 3). This provided for a relatively large area of contact between the 2 cell types. Incubation of mixtures for about 60 minutes at 37° C. was required for optimal adherence. The adherence was augmented not only by autologous serum but also by homologous sera and bovine fetal serum. Unabsorbed heterologous sera obtained from adult guinea pigs, mice, and rats usually caused clumping and lysis of rabbit cells. Heat inactivation and dialysis of sera against modified Hanks solution did not affect their ability to augment thymocyte-red cell adherence. In addition to sera, adherence was augmented by a variety of proteins consisting of rabbit albumin, gelatin, and rabbit gamma globulin at concentrations of 0.1 to one per cent. There was little or no adherence of rabbit thymocytes to dog, sheep, rat, mouse, and human red cells despite the presence of bovine fetal serum and incubation of cell mixtures at 37° C. for 60 minutes (Fig. 4).

In the presence of the serum or proteins capable of augmenting thymocyte-red cell adherence and incubation of the cell mixtures at 37° C. for 60 minutes,

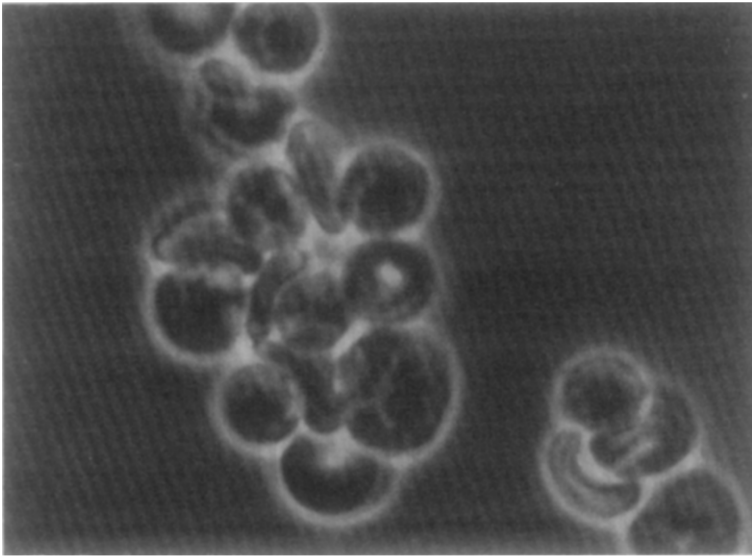


FIG. 3. Rabbit thymocytes and autologous red cells in modified Hanks solution containing 25 per cent autologous serum (phase photomicrograph). Mixtures were examined after incubation for 60 minutes at 37° C.

there was a significant degree of adherence of a proportion of rabbit lymph node lymphocytes to autologous red cells (Fig. 2, Column 4). Variability in the degree of the adherence of the lymph node lymphocytes to the red cells was large and was evident not only with lymph nodes of different rabbits but also with different lymph nodes obtained from the same rabbit. The adherence of lymph node lymphocytes to the red cells was in most cases less than the adherence of thymocytes of the same animal to the red cells.

Effect of Trypsin

Pretreatment of rabbit red cells with 0.01 to 0.5 per cent trypsin solutions completely abolished autologous thymocyte-red cell adherence which occurred in the absence of serum. The adherence of the red cells to thymocytes in the presence of serum could not be tested because trypsin-treated rabbit red cells agglutinated in the presence of sera. The ability of trypsin-treated thymocytes to adhere to red cells was likewise not determined because thymocytes agglutinated during trypsin treatment.

Effect of temperature and metabolic inhibitors

Thymocyte-red cell adherence which occurred in mixtures without incubation in 100 per cent salt solution was not significantly diminished by 0 to 4° C. temperature. It was likewise unaffected by a variety of metabolic inhibitors consisting of potassium cyanide, sodium fluoride, and iodoacetate solutions at concentrations of 5×10^{-4} to 5×10^{-2} M. In contrast, the augmentation of adherence which occurred in the presence of serum after incubation at 37° C. for 60 minutes was prevented by 0 to 4° C. temperature and 1.7×10^{-2} to 2.1×10^{-3} M iodoacetate

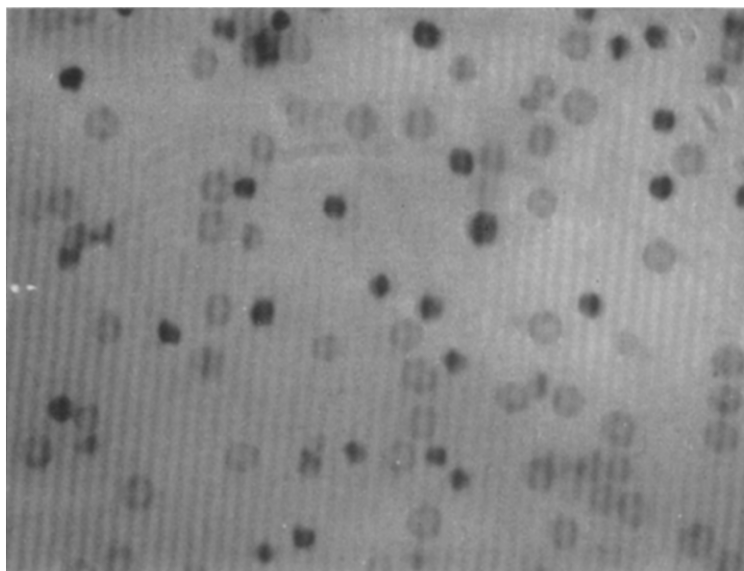


FIG. 4. Rabbit thymocytes and rat red cells were suspended in modified Hanks solution containing 25 per cent heat-treated fetal bovine serum and incubated at 37° C. for 60 minutes. Cells were fixed with formaldehyde vapor and stained with the Wright's stain.

TABLE I. Effect of iodoacetate upon thymocyte-red cell adherence

Iodoacetate concentration	Mean number of aggregates* per 100 high power microscopic fields (40x objective) $\pm$ standard deviation of 5 duplicate slides
$1.7 \times 10^{-2}M$	0 ± 0
$8.5 \times 10^{-3}M$	6 ± 2
$4.25 \times 10^{-3}M$	4 ± 3.8
$2.12 \times 10^{-3}M$	78 ± 26
$1.0 \times 10^{-3}M$	180 ± 25
$5 \times 10^{-4}M$	240 ± 40
0	280 ± 60

Cells were suspended in modified Hanks solution containing 15 per cent heat-treated fetal bovine serum and incubated at 37° C. for 60 minutes.

*Aggregates containing 6 or more cells were counted in 50 to 100 microscopic fields on each slide.

(Table I). The adherence was not affected by 5×10^{-4} to $5 \times 10^{-2}M$ potassium cyanide and sodium fluoride solutions. Pretreatment of thymocytes with $5 \times 10^{-3}M$ iodoacetate prevented the augmentation of adherence, but pretreatment of the red cells did not affect their ability to adhere to the thymocytes.

Newborn rabbits

The adherence of thymocytes obtained from newborn rabbits to autologous or homologous red cells exceeded the adherence of thymocytes obtained from adult rabbits to the red cells. This is illustrated in Table II. It can be seen that increased adherence occurred when thymocytes obtained from newborn rabbits were mixed with red cells which were obtained from either adult or newborn

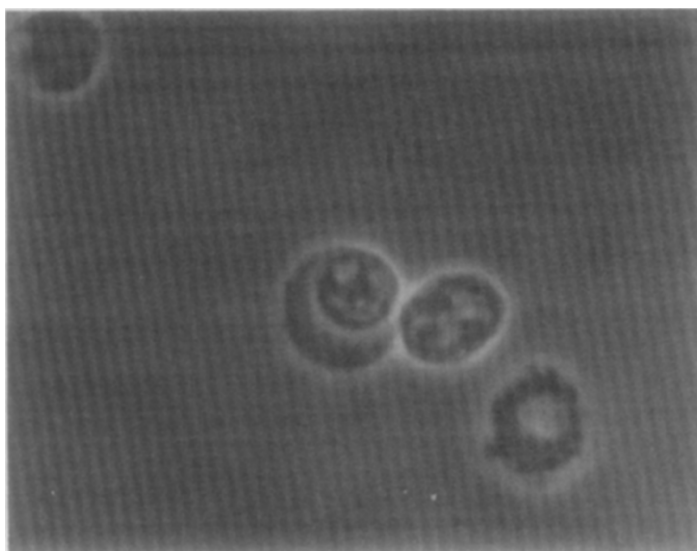


FIG. 5. Adherence of rabbit bone marrow erythroblast to autologous thymocyte (phase photomicrograph). Cells were suspended in modified Hanks solution containing 25 per cent autologous serum and incubated at 37° C. for 60 minutes.

TABLE II. Effect of age of rabbit upon rabbit thymocyte-red cell adherence

Source of thymocytes	Per cent of adhering thymocytes (mean $\pm$ S.D. of 5 duplicate slides)	
	Red cells from newborn	Red cells from adult
Newborn	74.9 $\pm$ 7.1	79.1 $\pm$ 7.1
Adult	42.3 $\pm$ 5.2	39.6 $\pm$ 5.0

Cells were suspended in 100 per cent modified Hanks solution and observed after sedimentation.

rabbits. The adherence of thymocytes obtained from adult rabbits to homologous red cells obtained from newborn rabbits was not significantly larger than the adherence of the thymocytes to red cells obtained from adult rabbits. This indicates that the increase in the adherence is due to the differences in the thymocytes rather than in the red cells.

Erythroblasts

Thymocytes adhered not only to mature red cells but also to nucleated red cells present in the bone marrow of adult and newborn rabbits and in spleens of newborn rabbits (Fig. 5). The adherence was augmented by conditions which augmented thymocyte-mature red cell adherence and exceeded the adherence of extrathymic lymphocytes to the erythroblasts.

Thymocyte-red cell adherence in other species

Preliminary results are illustrated in Table III. A relatively large degree of autologous or homologous thymocyte-red cell adherence occurred not only in the

TABLE III. Adherence of thymocytes to homologous and heterologous red cells

Source of thymocytes	Per cent of adhering lymphocytes (mean $\pm$ S.D. of 5 duplicate slides)			
	Red cells from rabbit	Red cells from mouse	Red cells from rat	Red cells from guinea pig
Rabbit	87.8 $\pm$ 4.1	1.2 $\pm$ 2.2	4.9 $\pm$ 8.25	2.3 $\pm$ 2.8
Mouse	8.8 $\pm$ 5.1	6.3 $\pm$ 5.4	62.6 $\pm$ 13.0	13.5 $\pm$ 8.1
Rat	0.3 $\pm$ 0.56	1.7 $\pm$ 1.0	95.7 $\pm$ 2.24	2.7 $\pm$ 1.4
Guinea pig	100 $\pm$ 0	1.7 $\pm$ 2.6	0 $\pm$ 0	0 $\pm$ 0

Cells were suspended in modified Hanks solution containing 15 per cent heat-inactivated bovine fetal serum and incubated at 37° C. for 60 minutes.

rabbit but also in the rat. This occurred in 100 per cent modified Hanks solution but was augmented by autologous or bovine fetal serum. There was thymocyte-red cell adherence in heterologous mixtures containing guinea pig thymocytes and rabbit red cells or mouse thymocytes and rat red cells. All Lymphocyte-red cell autologous and heterologous adherences were greatly diminished when lymph node lymphocytes were utilized instead of thymocytes.

DISCUSSION

The main findings in this study are the observations that rabbit and rat thymocytes have an affinity for autologous red cells. This affinity is sufficiently high to cause the cell types to adhere to each other almost immediately upon sedimentation of the cell mixtures. The adherence is evident despite shearing forces which tend to disperse the aggregates during resuspension of the sediment by a pipette.

The fact that there is little or no adherence in most heterologous combinations indicates that the adherence is mostly species specific. This specificity cannot be accounted for by assumptions which attribute the adherence to the general adhesiveness of the cells. The specificity can best be explained by the assumption that it results from the interactions of specific molecular groupings (receptors) present upon the surfaces of the red cells and the thymocytes.

The receptors are part of or are firmly attached to the cell surfaces. This is suggested by the fact that thymocyte-red cell adherence occurred despite the fact that the cells had been washed and suspended in 100 per cent modified Hanks solution. The synthesis of a significant quantity of soluble factors is unlikely since adherence in the absence of serum required little or no incubation of the washed cell mixtures. The fact that pretreatment of the red cells with relatively small concentrations of trypsin abolished thymocyte-red cell adherence indicates that the red cell receptors are part of or are dependent upon the presence of surface protein molecules.

The thymocyte receptors are affected by age-dependent developmental processes. Thus, thymocytes of newborn rabbits adhere to a larger extent to rabbit red cells than do thymocytes of mature rabbits. The red cell receptors likewise develop at a relatively early stage of the red cell maturation process, since thymocytes adhere not only to mature red cells but also to nucleated red cells.

The fact that iodoacetate, a known inhibitor of the glycolytic system, inhib-

its the augmentation of adherence suggests that the augmentation is dependent upon energy produced by the cells. This is consistent with the fact that optimal augmentation requires incubation at 37° C. and does not occur when the cells are incubated at 0 to 4° C. The active cell type during augmentation of adherence is the thymocyte, since pretreatment of the thymocytes with iodoacetate inhibits the augmentation while pretreatment of the red cells does not. The adherence of thymocytes to red cells in the absence of serum does not require active thymocytes, since it is not significantly affected by conditions which inhibit enzymatic activity.

In addition to thymocytes, rabbit lymph node lymphocytes adhere, under certain conditions, to autologous red cells. Several differences between the adherences of lymph node lymphocytes and thymocytes to autologous red cells are, however, evident. Significant lymphocyte-red cell adherence occurs in 100 per cent modified Hanks solution in most thymocyte-autologous red cell mixtures but not in most lymph node lymphocyte-autologous red cell mixtures. The adherence of lymph node lymphocytes to autologous red cells in the presence of sera or other proteins occurs in most cases to a lesser extent than in mixtures containing thymocytes of the same animal instead of lymph node lymphocytes. The physiologic factors which determine the degree of lymphocyte-autologous red cell adherence require further investigation. One factor controlling the degree of adherence may be the state of maturation of the thymocyte. This is indicated by the fact that the adherence of thymocytes obtained from newborn rabbit to autologous red cells usually exceeds the adherence of thymocytes obtained from mature animals.

Selective autologous adherence is essential for the formation of tissues and organs in multicellular organisms.⁸ This occurs through developmental processes which are not known to be associated with the immune system of the organism. The adherence of thymocytes to autologous and heterologous red cells, under conditions reported in this study, may likewise occur through developmental rather than immune processes. This is suggested by the fact that the adherence of thymocytes to the red cells, in most cases, exceeds the adherence of lymph node lymphocytes to the red cells. Lymph node lymphocytes are usually more active than thymocytes during immune responses. In addition, a relatively large proportion of thymocytes adhere to one autologous or heterologous cell type. If this represented an immune response, it would preempt (according to clonal selection theories) most of the thymocytes required for specific responses to unrelated antigens.

The functions of the adherence of lymphocytes to autologous red cells are unknown. The following may, however, be considered. One of the main functions of the thymus is the seeding of peripheral lymph node organs with lymphocytes.⁹ Thymocytes enter the vascular system through the medullary veins in the thymus.¹⁰ These thymocytes will probably adhere to circulating red cells *in vivo*, since the adherence is augmented by the presence of autologous serum. This adherence is likely to prolong the circulation of the thymocytes in the vascular system and hinder their exit into the peripheral lymphoid organs. The adherence may thus participate in homeostatic mechanisms which

limit the migration of thymocytes to peripheral lymphoid organs. Differences between the adherences of lymph node lymphocytes and thymocytes to circulating autologous red cells may help separate the homing patterns of the 2 cell types. The adherence of thymocytes to certain heterologous red cells may be a fortuitous phenomenon resulting from cross-reactivity of the red cells with thymic surface receptors concerned with autologous affinity. This, rather than cellular immunity alone, may account, in part, for the adherence of thymocytes and peripheral lymphocytes of untreated human subjects to sheep red cells.¹¹⁻¹³

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REFERENCES

- 1 Granger, G. A.: *In* Lawrence, H. S., and Landy, M., editors: Mediators of cellular immunity, New York, 1969, Academic Press, Inc., p. 324.
- 2 McFarland, W., and Heilman, D. H.: Lymphocyte foot appendage: Its role in lymphocyte function and in immunological reactions, *Nature* **205**: 887, 1965.
- 3 Siegel, I.: Autologous macrophage-thymocyte interactions, *J. ALLERGY* **46**: 190, 1970.
- 4 Siegel, I.: Natural and antibody-induced adherence of guinea pig phagocytic cells to autologous and heterologous thymocytes, *J. Immunol.* **105**: 879, 1970.
- 5 Siegel, I.: The selective adherences of thymocytes to autologous rabbit red cells and autologous mast cells, *J. Cell Biol.* **47**: 191a, 1970.
- 6 Martin, S. P., and Green, R.: Methods for the study of surviving leukocytes, *in* Warren, J. V., editor: *Methods in medical research*, vol. 7, Chicago, 1958, Year Book Medical Publishers, Inc., p. 136.
- 7 Bessis, M.: *Cytology of the blood and blood forming organs*, New York, London, 1956, Grune & Stratton, Inc.
- 8 Moscona, A. A.: Recombination of dissociated cells and the development of cell aggregates, *in* Willmer, E. N., editor: *Cells and tissues in cultures*, vol. 1, New York, 1960, Academic Press, Inc., p. 489.
- 9 Burnet, M.: *Cellular immunology*, London, 1969, Cambridge University Press.
- 10 Sainte-Marie, G., and Leblond, C. P.: Thymus cell population dynamics, *in* Good, R. A., and Gabrielson, A. E., editors: *The thymus in immunobiology*, New York, 1964, Harper & Row, Publishers, p. 207.
- 11 Wybran, J., and Fudenberg, H.: Rosette formation: a test for cellular immunity, *Trans. Assoc. Am. Physicians* **84**: 239, 1971.
- 12 Bach, J. F., Dormont, J., Dardenne, M., and Balner, H.: *In vitro* rosette inhibition by anti-human antilymphocyte serum, *Transplantation* **8**: 265, 1969.
- 13 Brain, P., Gordon, J., and Willets, W. A.: Rosette formation by peripheral lymphocytes, *Clin. Exp. Immunol.* **6**: 61, 1970.