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14. I would appreciate the gift of such fibers and also of samples of α-keratins from the Pleistocene.

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Antigen-Binding Cells in Normal Mouse Thymus

Abstract. *The thymus of a normal adult mouse contains lymphocytic cells with a large number of antigen-binding receptors. Binding of the antigen by these cells is specific and can be inhibited by cross-reactive materials. It is possible that the interaction of thymocytes with antibody-forming precursor cells, required in the primary immune response to certain antigens, is mediated by these specific antigen-binding cells of the thymus.*

In the mouse, precursors of antibody-forming cells interact with thymus-derived, antigen-reactive cells (ARC) to proliferate and differentiate into hemolysin-producers (*1, 2*). Thymus cells respond to antigenic stimulation with immediate proliferation (*3*), but without antibody formation (*4*). Recognition of antigen by cells is believed to be mediated by antigen receptors, presumably antibody molecules (*5*). Hence ARC of thymus, if specific, should be capable of binding the antigen. Naor and Sulitzeanu (*6*) have demonstrated the presence of specific, antigen-binding cells in the spleen and lymph nodes of normal mice by their sensitive autoradiographic technique. However, antibody-forming cells to many antigens have been demonstrated in the spleen (*7*), but not in the thymus (*8*) of normal animals. We examined the thymus of normal mice for presence of antigen-binding cells (presumably ARC and not background antibody-forming cells) by immunofluorescence and A-H assay. The A-H assay (an acronym for antigen binding to cells determinated by enzymatic fluorogenic group hydrolysis) (*9*) utilizes β-galactosidase of *Escherichia coli* (βGz) as antigen and is based on the measurement of the enzymatic activity of cell-bound βGz by Rotman's method (*10*) which allows the detection of the activity of single molecules of this enzyme. Accordingly, about 10^8 washed cells from A/Jax mice (8 to 10 weeks old) were incubated with 15 to 50 μg of βGz at 37°C for 1 hour or at 4°C overnight. As a control, a portion of each cell suspension was incubated without enzyme. After the incubation, the cells were washed four times each with 20 ml of Hanks balanced salt solution (HBS) at 4°C to remove unbound enzyme. These cells will be referred to as "prepared" cells. A sample was removed, and nucleated cells were counted in a hemacytometer. Cell suspensions were then diluted in HBS to obtain the desired number of cells (i) for determination of total antigen-binding activity; (ii) for enumeration of active cells and measurement of their activity; and (iii) for morphological studies by the immunofluorescence technique.

To determine total activity, 0.2 ml of a cell suspension containing approximately 1×10^7 to 2×10^7 cells was mixed with 0.2 ml of $4.8 \times 10^{-5}M$ fluorescein di-β-galactopyranoside (FDβG), and the intensity of the fluorescence produced during 6 hours at room temperature was measured by a Turner fluorometer (model 111, equipped with 47B and 2A-12 filters). The results (Table 1) show that 10^7 thymus cells from normal mice bind considerably more βGz than do the same number of spleen cells. To determine the specificity of βGz binding, the cell suspensions were incubated with 5.0 mg of heat-inactivated βGz at 37°C for 1 hour before the active enzyme was added. Heated enzyme (56°C for 10 minutes) is antigenically cross-reactive but enzymatically inactive. In the presence of Mn^{2+}, Mg^{2+}, and reducing agents, the heated enzyme may regain up to 30 percent of its original enzymatic activity after 10 to 12 hours. Hence, in experiment 3, the overnight incubation was omitted. Some binding of the enzyme by thymus and spleen cells is specific and can be inhibited by excess heated enzyme. Presence of 5 percent fetal calf serum or other unrelated proteins has little or no effect.

Table 1. Antigen-binding activity of normal mouse thymus and spleen cells. Results are expressed as the increase in fluorometer units per 10^7 nucleated cells. In experiment 3, the cells were first incubated for 1 hour at 37°C with 5.0 mg of heated βGz (inactive enzyme) and then for an additional hour with 50 μg of active βGz. They were washed after the second incubation period and assayed together with the test samples, which were not exposed to inactive enzyme.

Cell	Experiment 1	Experiment 2	Experiment 3	
			Test sample	Inactive enzyme + βGz
Thymus	10.7	11.0	12.2	3.9
Spleen	4.0	3.5	6.8	3.1

For enumeration of active cells in a population, a modified A-H assay was used. Accordingly, "prepared" cells were diluted with HBS to contain approximately 5×10^6 cell/ml. A portion was mixed with an equal volume of $4.8 \times 10^{-5}M$ FDβG, and hundreds of 0.2-μl droplets were placed in oil chambers with a "Terasaki Dispensor" (Hamilton Co., Whittier, Calif.). Each droplet, therefore, contained approximately 500 cells. In the course of a 30- to 60-minute incubation at 37°C, fluorescent droplets with considerable activity could be visualized by the naked eye when the chamber was illuminated by a blue light. Each active droplet was then examined under a microscope, and the ones with clumps were disregarded. The activity of the droplets was measured by an Aminco photomultiplier-microphotometer attached to a Zeiss fluorescence microscope (*9*). Since the frequency of active droplets is usually less than 1×10^{-2}, the probability that each active droplet contains more than one active cell is low. Based on this reasoning, the frequency of active cells in normal mouse spleen and thymus was calculated (Table 2). The frequency of active cells in the normal thymus is greater than that of those in the spleen. This is in contrast to the findings of Byrt and Ada (*11*) in which antigen-binding cells are more frequent in spleen than thymus. This discrepancy, however, may be due to the different antigens used in the two studies and to the fact that only the cells which

absorbed at least 1.2×10^5 molecules of βGz were considered active.

For morphological studies, the fluorescent antibody technique was employed. Hence, "prepared" cell suspensions were fixed with 95 percent ethanol and incubated at 37°C for 2 hours with the globulin fraction of rabbit antiserum to βGz conjugated to fluorescein isothiocyanate (FITC). The cell suspensions were then washed three times with 0.15*M* phosphate-buffered saline (*p*H 7.2), and smears were made for observation under a Zeiss fluorescence microscope. As a control, FITC-labeled globulin fraction of rabbit antiserum to keynote limpet hemocyanin, and FITC-labeled globulin fraction of rabbit antiserum to mouse gamma globulin (MGG) were incubated with "prepared" as well as control cells (without βGz).

Among the few cells that reacted with antiserum to βGz (13 total, observed in 18 slides of the test samples), three morphologically distinct types could be discerned: a small thymocyte (Fig. 1A), a medium-size thymocyte with considerable cytoplasm (Fig. 1B), and a large, diffusely stained, mononuclear cell (Fig. 1C). However, in the cell suspension stained with antiserum to MGG, two reactive cells (Fig. 1D) were observed which were morphologically similar to the third type mentioned above. In addition, in one experiment, a few typical plasma cells reacted strongly with antiserum to MGG. Except for cells which were stained with antiserum to MGG, no reactive cell was present in any of the control samples. It is noteworthy that the small thymocytes which contained antibody to βGz appeared with distinct fluorescent areas (Fig. 1A). This may be due to the presence of clusters of antigen receptors on the cell surface.

Specific antigen-binding cells have also been detected by DeLuca *et al.* (*12*) in normal mouse thymus and bone marrow and in normal bone marrow of rabbits. Ada and Byrt have shown that damage to specific antigen-binding thymocytes suppresses the response to that antigen specifically (*13*). Thymus, however, lacks the "background" plaque-forming cells and even after immunization does not contain antibody producers (*8*). In fact, soon after immunization, the antigen-binding activity of the thymus decreases when spleen becomes more active. However, direct migration of active cells from the thymus to spleen after antigen immunization, without considerable change of antigen receptors, is ruled out (*14*).

Table 2. Antigen-binding cells of normal mouse thymus and spleen. The number of antigen-binding cells (ABC) was calculated based on the assumption that each active droplet contained only one active cell.

Experiment	Droplets (No.)	Average cells per droplet	Active droplets	ABC per 10^6 nucleated cells	Average activity (molecules per cell)
			Thymus		
1	132	580	8	103	
2	116	560	6	92	280,000
3	520	580	8	27	
			Spleen		
1	89	660	2	34	
2	245	560	3	21	120,000
3	580	650	4	11	

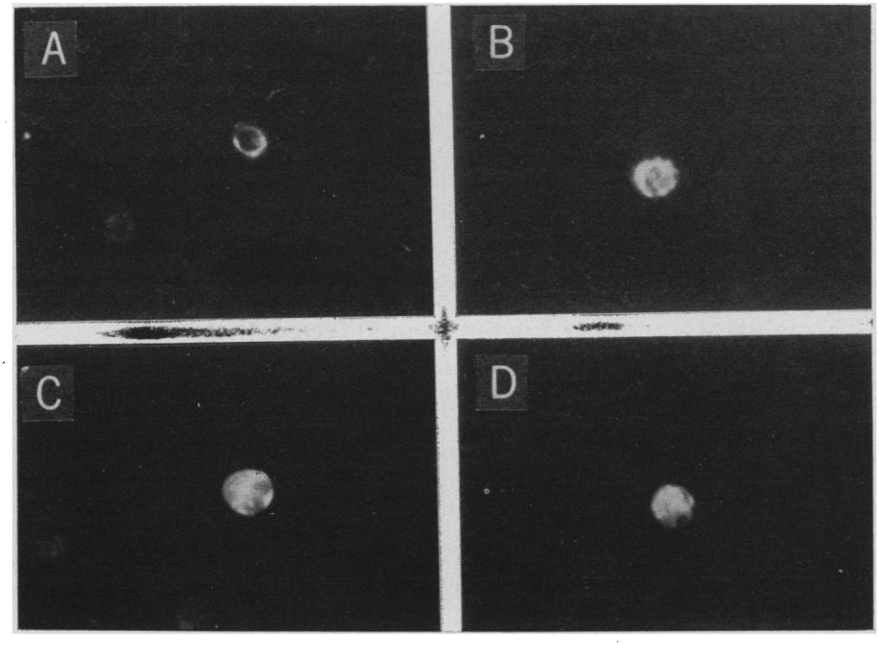

Fig. 1. Fluoromicrographs of normal mouse thymus cells (× 500). (A to C) Cells stained with fluorescein-labeled globulin fraction of rabbit antiserum to βGz; and (D) cells stained with fluorescein-labeled globulin fraction of rabbit antiserum to MGG antibody. Before staining all cells were incubated with βGz.

Hence the specific antigen binders of the thymus reported here are most likely the antigen-reactive cells of Claman *et al.* (*1*) and Miller and Mitchell (*2*) and are most likely the small and medium-size lymphocytes shown in Fig. 1, A and B.

It is known that neonatal thymectomy leads to impairment of immunological development (*15*). The effects of thymectomy on the specific immune response in adults, however, can not be detected immediately (*16*) but become apparent 6 to 9 months after the operation (*17*). Presumably by this time the thymus-derived cells present in other lymphatic organs are exhausted. Some of the antigen-binding cells in lymphatic organs may be thymus-derived ARC. Metcalf and co-workers (*18*) have shown that the number of small lymphocytes decrease in the spleen, the lymph nodes, and the circulation after adult thymectomy. Thymectomy in adults also has been reported to prevent or delay both regeneration of lymphoid organs following x-irradiation (*19*) and recovery from specific immunological paralysis (*20*). All of these authors point out that the thymus is involved in the development and maintenance of immunological responsiveness. Direct identification of antigen-binding cells reported here explains these findings and indicates that one function of the thymus may be mediated by these cells. The exact mechanism of cellular interaction of the immune response, however, remains unclear.

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Inflammation and Herpes Simplex Virus: Release of a Chemotaxis-Generating Factor from Infected Cells

Abstract. *Infection of primary rabbit kidney cells with herpes simplex virus leads to the release of a cell factor or factors that upon incubation with serum results in the cleavage of the fifth component, C5, of complement. The product of this cleavage, C5a, is chemotactic for polymorphonuclear leukocytes and could be responsible for the accumulation of these cells at the site of herpetic lesions.*

Although inflammatory cells are often seen at the site of virus-induced tissue destruction (*1*), little is known about the factors responsible for the local accumulation of these cells. It has been postulated that intracellular products released from damaged parenchymal cells might serve as stimulants for inflammation (*2*), but it has been difficult to isolate and characterize these products from in vivo infections. An in vitro system, however, would make it possible to dissect the inflammatory response and isolate the factors released from virus-infected cells. In addition, the ability of these factors to interact with serum components and to specifically attract inflammatory cells could be determined quantitatively by the leukocyte migration technique (*3*). We now describe the release in vitro of a polymorphonuclear leukocyte chemotaxis-generating factor (or factors) from cultures of primary rabbit kidney cells infected with herpes simplex virus and the interaction of this factor with serum complement.

Monolayers of primary rabbit kidney cells (PRK) and stock pools of herpes simplex virus (HSV, type 1) were prepared as described (*4*). Gey's medium or Gey's medium mixed with an equal volume of Eagle's medium (*5*) were used as the diluents. To study the factors released from virus-infected cells, PRK monolayers were infected with HSV diluted in the Eagle-Gey medium. After 3 hours, the inoculum was aspirated; the monolayers were then washed three times and incubated with a fresh portion of the Eagle-Gey medium at 37°C in humidified atmosphere containing 5 percent CO_2. At various times the used medium was removed from the cells and centrifuged at 2000*g* for 10 minutes; the supernatants were separated and stored at −70°C. To test for chemotactic activity, supernatants were incubated alone or with rabbit or guinea pig serum (37°C for 60 minutes) (*6*, *7*). These mixtures then were assayed for chemotactic activity by measuring migration of polymorphonuclear leukocytes (PMN's) through a micropore filter (1.2 μm) by a modification of the Boyden technique (*8*).

To see whether uninfected PRK cells contained factors capable of initiating a chemotactic response, approximately 3×10^7 cell/ml in Gey's medium were sonicated for 2 minutes (4°C) and centrifuged at 10,000*g* for 30 minutes (4°C); the supernatants (uninfected cell lysates) were then tested for chemotactic activity. Table 1 shows that lysates alone had no significant chemotactic activity. Incubation of lysates with homologous rabbit serum, however, resulted in a marked increase in activity. Only a slight increase in chemotactic activity occurred when the lysates were incubated with serum that had been heated at 56°C for 30 minutes. Addition of ethylenediaminetetraacetic acid to the incubation mixture inhibited generation of chemotactic activity, whereas soybean trypsin inhibitor had no significant effect. These experiments show that a chemotaxis-generating factor (CGF) is present in lysates of uninfected PRK cells (*9*).

To see whether HSV infection of PRK cells would result in the release of CGF, PRK monolayers (2×10^6 cells) were infected with 4×10^6 plaque-forming units (PFU) of HSV. At various times thereafter supernatants were removed and tested for CGF activity. Uninfected monolayers served as controls. Release of CGF occurred within 12 hours after infection (Fig. 1) and was associated with the appearance of cytopathology. At the time of maximum release from the infected monolayers, neither release nor cytopathology was detected in uninfected monolayers. The conditions required for the produc-

Table 1. Chemotactic activity generated in rabbit serum by uninfected cell lysate and infected cell supernatant. One milliliter of uninfected cell lysate or infected cell supernatant (collected 22 to 24 hours after infection) was incubated at 37°C for 60 minutes with 1.0 ml of the appropriate reagent, and 0.5 ml of each reaction mixture was assayed for chemotactic activity. PMN, polymorphonuclear leukocyte; EDTA, ethylenediaminetetraacetic acid.

Reagents added	Chemotactic activity (PMN's per high-power field)	
	Uninfected cell lysate	Infected cell supernatant
None	11	8
Serum	185	168
Heated (56°C; 30 minutes) serum	28	40
0.012*M* EDTA (*p*H 7.2) plus serum	32	41
Soybean trypsin inhibitor (5 mg/ml) plus serum	210	144
Serum alone without lysate or supernatant	14	17