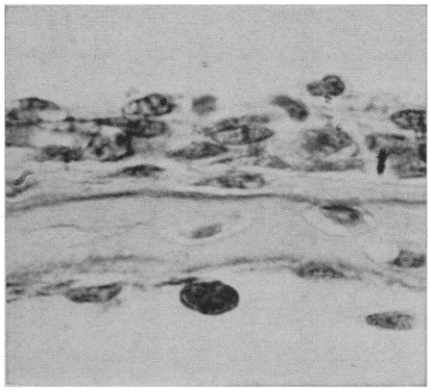

Fig. 3. High magnification of the endosteum, demonstrating the lack of distinct intercellular margins within the cytoplasm. (× 900)

tected, but these assays did not provide definitive results concerning the endosteum. A very slight reaction for alkaline phosphatase was seen in the endosteal area, while a much greater reaction was present in the osteoblastic cells of the periosteum. The outermost, fibrous periosteum, on the other hand, was unreactive. Succinic dehydrogenase was present in all the embryonic bone cells and did not appear concentrated in any particular area. (The succinic dehydrogenase reaction was monitored by noting its activity in a piece of muscle adjacent to the bone.) The distribution of all these enzymes in the bone of the chick embryo appears to be qualitatively similar to that seen in other animals, although there may be quantitative differences (*3*, *4*).

Additional evidence concerning the nature of the medullary lining was provided by autoradiography after the administration of tritiated thymidine.

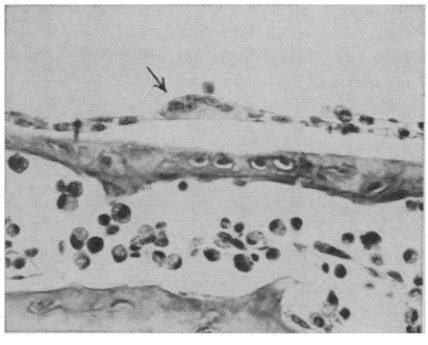

Fig. 4. Longitudinal section of a femur from a chick embryo aged 17 days. Orientation as in Fig. 1. A small osteoclast (arrow) is in continuity with the endosteum. (× 370)

Very few of the nuclei within the multinucleated endosteum were labeled 1 hour after the injection of isotope. By contrast, numerous nuclei of the marrow, periosteum, and cartilage cells were labeled at this time. This difference is in agreement with previous studies (*5*) which indicate that osteoclasts incorporate tritiated thymidine considerably later than other bone cells.

The results of our experiments are consistent, and indicate that rapid bone removal takes place in the 14- to 17-day chick embryo and that such removal is probably due to osteoclastic activity of the endosteum. Contrary to these conclusions, expansion of the marrow cavity due to erosion of the inner bone spicules has not been thought to occur during osteogenesis in the avian embryo (*6*), although continuous removal of the preceding cartilage by erosion from within has been recognized for many years. Perhaps the sparseness of typical osteoclasts has fostered the concept of insignificant bone resorption in these embryos. In addition to the other studies, we also examined fresh tibias from chick embryos aged 14 to 17 days, and observed a considerable widening of the marrow cavity with increasing age (*2*).

Although our results differ from previous concepts concerning the occurrence of bone resorption during osteogenesis in the chick embryo, they are in complete agreement concerning the process of bone deposition. As previously described (*1*, *6*), we did not see any histological evidence of endochondral ossification until very late in embryogenesis (about day 19). Our autoradiographic results confirm the fact that subperiosteal and subperichondral bone formation are the major sources of new bone in these embryos.

The apparent lack of distinct intercellular membranes within the multinucleated endosteum, the occasional presence of typical osteoclasts in continuity with it, and its apparent ultimate transformation into numerous, small osteoclasts suggest that the endosteum may represent a network of fused primordial osteoclasts acting in coordination. The relative lack of typical deep Howship's lacunae can probably be attributed to uniform erosion of the spicule surface by the covering cell layer. The symmetrical deposition, continued linear uniformity, and symmetrical removal of the labeled collagen laminae imply a well-coordinated structural and functional organization of bone in the chick embryo. It is conceivable that there is some regulating mechanism which closely synchronizes the activities of the periosteal and endosteal regions (*7*).

MARVIN L. TANZER
RONALD D. HUNT
U. S. Army Medical Research and Nutrition Laboratory, Fitzsimons General Hospital, Denver, Colorado

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Circulating Small Lymphocytes: Immunologically Competent Cells with Limited Reactivities

Abstract. *Highly purified small lymphocytes from peripheral blood are capable of producing acute transplantation disease. However, such lymphocytes do not transfer from sensitized to normal mice the capacity to produce antibody to bovine serum albumin under conditions where mixed populations of lymphoid cells from the sensitized donors are effective.*

There is now compelling evidence that purified, small circulating lymphocytes are capable of producing acute transplantation (that is, graft-versus-host) disease in mammals (*1*, *2*). After injection, such adult lymphocytes or their immediate descendants promptly attack antigenically disparate newborn hosts and mediate graft-versus-host reactions that constitute a model of immunologic disease (*3*). Moreover, small lymphocytes from adult mice may protect isogenic newborn mice against transplantation disease (*4*). The mechanisms by which these lymphocytes bring about immune reactions—whether cellular, humoral or both—are not clear.

In our experiments, lymph node and spleen cell populations readily transferred production of humoral antibody to isogenic mouse recipients, whereas small blood lymphocytes completely failed to do so under the same conditions.

Recent experiments (*5*) indicate that adult mice of the A/Jax inbred strain may be effectively sensitized to bovine serum albumin (BSA) and that persistent antibody production as measured by the sensitive Farr test (*6*) may then be transferred by lymph node and spleen cells to normal recipients of the same strain. An initial subcutaneous injection of 0.5 mg of BSA in complete Freund's adjuvant (Difco) followed by intracutaneous booster injections of 0.1 mg of BSA in normal saline on days 17 and 24 is adequate to induce specific antibodies in substantial titer by the 26th day. Pooled serum from these animals revealed sufficient antibodies even at a 1:640 dilution to precipitate, in standardized tests, 50 percent of BSA labeled with I^{131}. When 1 million or more viable lymphoid cells were harvested from these animals on day 26 and transferred intraperitoneally to normal adult recipients, specific antibody production was demonstrable for about 3 weeks thereafter. The timing of the cell transfers was found to be near optimum relative to the appearance of antibodies and Arthus skin reactions in the donors. Preparations of frozen and thawed cells failed to transfer measurable antibody or to sensitize recipient mice actively to BSA. The same experimental design was applied to determine whether purified small lymphocytes from A/Jax mice immunized with BSA are capable of transferring antibody production under the conditions where mixed populations of lymphoid cells were effective.

After immunization with BSA, the blood from 42 mice was collected in sufficient heparin to prevent clotting. The whole blood and 6-percent citrated-bovine fibrinogen (Armour) in normal saline were then mixed in equal volumes and incubated at 37°C for about 20 minutes to sediment the erythrocytes. The leukocytes in the supernatant were washed six times in cold Hanks balanced salt solution to remove the fibrinogen; they were then slowly filtered twice through columns of tightly packed glass wool to remove the granulocytes and large mononuclear cells. Differential cell counts revealed that 99 percent (out of 2300 leukocytes counted in the final preparation) were small lymphocytes. Dye-exclusion tests with Eosin-Y showed that 99 percent of these lymphocytes were viable. The final lymphocyte preparation was counted and diluted in Hanks balanced salt solution to provide separate suspensions containing 100,000, 1 million, and 10 million cells, respectively. Control preparations of nonviable lymphocytes were in turn prepared by repeated freezing and thawing. Each of five groups of normal, adult, male A/Jax mice was injected intraperitoneally with one of these cell suspensions. At 2, 5, 12, and 19 days thereafter, serum samples were obtained from these recipients and tested for antibodies to BSA. The results are given in Table 1. The quantity of specific antibody present was indicated by the percentage of binding capacity for BSA antigen labeled with I^{131} in the test serums. All dilutions were made in normal A/Jax mouse serum diluted 1:10 with borate buffer (*6*). The I^{131}-BSA precipitable with pooled, normal, mouse serums ranged from 8 to 10 percent; values less than 12 percent indicate absence of specific antibodies to BSA (*5*). Although lymph node and spleen cells could transfer the production of antibodies, neither viable nor frozen-thawed small lymphocytes in comparable numbers did so.

Table 1. Antigen (BSA) binding by serums, diluted 1:5, from adult A/Jax mouse recipients of lymphoid cells transferred from isogenic donors immunized with BSA. Numbers in parentheses indicate number of mice contributing to the test serum pool.

Cell inoculum transferred*	No. of recipients	Antigen precipitated (%) at times after cell transfer†			
		2 days	5 days	12 days	19 days
10^6 to 10^7 viable lymph node cells	24	6 (6)	8 (6)	22 (6)	25 (6)
10^8 viable lymph node cells	8	26 (2)	98 (2)	99 (2)	99 (2)
10^8 spleen cells	9	25 (2)	73 (2)	89 (3)	78 (2)
10^7 viable small lymphocytes	3	10 (1)	9 (1)	14 (1)	—
10^6 viable small lymphocytes	20	9 (5)	10 (5)	9 (5)	9 (5)
10^6 frozen-thawed small lymphocytes	18	9 (4)	9 (5)	10 (5)	10 (4)
10^5 viable small lymphocytes	25	11 (6)	9 (7)	10 (6)	10 (6)
10^5 frozen-thawed small lymphocytes	20	9 (5)	10 (5)	9 (5)	8 (5)

* Pooled donor group serums at 1:640 dilution precipitated about 50 percent of I^{131}-BSA in Farr tests, indicating substantial antibody titers at the time lymphoid cells were harvested. † Values for pooled normal mouse serums ranged from 8 to 10 percent; values less than 12 percent indicate absence of BSA antibodies.

The possibility of slight antibody production 12 days after transfer of 10^7 viable small lymphocytes may reflect the presence of larger, proliferating lymphocytes as contaminants (approximately 1 percent) in higher dosage. It should be noted that the highest dosage, 10^8, of lymph node or spleen cells was most effective in the transfer of antibody production. Since lymph nodes and spleen contain heterogeneous populations of nucleated cells, it would be expected that many of the transferred cells were not engaged in production of BSA antibodies. Lymphoid cells in these populations were effective in lower dosage, but significant antibody production was not detectable until about 12 days after transfer. Additional control experiments ruled out the possibility that transfer of previously formed antibody or of retained antigen was responsible for the positive results obtained (*5*).

Although 900,000 or more small lymphocytes are sufficient to produce fatal transplantation disease (*1*), even ten times as many of these cells were incapable of transferring humoral antibody production in our experiments. The evidence suggests that a subpopulation of circulating small lymphocytes is effective in transplantation immunity, but it does not give rise to cells that elaborate typical humoral antibodies. One might consider the possibility that circulating lymphocytes settle down in lymph nodes and spleen once they become committed to a particular immune response; one would then not expect to find these cells circulating in substantial numbers after antibody was being produced. Insofar as capacity to produce transplantation disease is concerned, this possibility has been refuted (*1*). The qualitatively different behavior of small lymphocytes in the two test systems investigated would have been obscured by the use of heterogeneous lymphoid cell populations (*7*). Other aspects of this problem require further study: lymphocyte population kinetics

in mice after prolonged immunization with BSA are almost certainly different from those associated with acute transplantation disease; moreover, quite different chemical and biological tests with a purified protein antigen and complex isoantigens, respectively, are compared.

Although current thinking is dominated by the inferential assumption that a sequence, lymphocyte → plasmocyte → humoral antibody, affects nearly all immune reactions, we are attracted to the hypothesis that cellular immunity—whether enzymic or referable to a separate class of endoantibodies—is associated with small lymphocytes. Other recent studies (*8*) suggest that lymphocytes and histiocytes function as specifically immune cells under conditions where serum antibodies alone are ineffective or actually interfere with cellular immunity. Since functional heterogeneity in populations of small lymphocytes is apparent (*2, 9*), it is probably erroneous to equate function with cell morphology as such. Moreover, small lymphocytes appear to be deficient in cytoplasmic organelles such as lysosomes that could be expected to cause the digestion or dissolution of antigen or other cells. Thus the supposition that a small lymphocyte has to turn into something different before it engages in immunological responses continues to be attractive (*10*).

DONNA L. VREDEVOE*
W. H. HILDEMANN†
Department of Medical Microbiology and Immunology, University of California, Los Angeles

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* Present address: Department of Medical Microbiology, Stanford University, Stanford, Calif. † Present address during sabbatical leave: Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

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Isolated Mammalian Eye: A Method for Quantitative Evaluation of Autonomic Drugs

Abstract. *The enucleated rodent eye can be mounted for study in vitro, in a chamber that contains electrodes suitably placed to enable the severed postganglionic nerves to the iris to be stimulated. The technique provides a simple quantitative method for the study of drugs which act at the parasympathetic neuro-effector junction.*

For many years the iris has been used as a pharmacological tool in the analysis of drug action at the autonomic neuro-effector junction. Studies of the mammalian eye in vitro have been confined to experiments with isolated excised iris, iris strips, or iris segments (*1*). In numerous other studies, the pupillary diameter of the eye has been measured *in situ* after local or systemic administration of a drug (*1, 2*). While the results of these experiments have been informative, the isolated iris is relatively fragile, not easily prepared, and its response is not easily determined quantitatively.

Recently, a simple method was described by which drug effects can be evaluated quantitatively by measuring the pupillary diameter of intact eyes enucleated from either the rat, mouse, or guinea pig (*3*). The reactivity to drugs of this pupillary neuro-effector system in vitro was found to be remarkably stable and reproducible. While the technique is applicable to adrenergic drugs, it offers particular advantages in assessing drug action at the parasympathetic neuro-effector site. It is free of the interpretive problems inherent in the use of such systems as the isolated gut with its included ganglion cells and intrinsic innervation; and it overcomes the objection to methods such as measurement of blood pressure, contraction of the urinary bladder, or salivary secretion in the intact animal, where the significance of results is compromised by uncertainties relative to varying drug distribution, drug elimination, and homeostatic mechanisms. It provides a simple quantitative method that permits maintenance of drug concentration, preserves the integrity and reactivity of the mammalian parasympathetic pupillary mechanism, and eliminates extraneous stimuli. This method has now been extended to include electrical stimulation of the postganglionic parasympathetic and sympathetic nerves which control pupillary size.

As before, the animals were killed and the eyeballs were enucleated with blunt curved surgical scissors, care being taken to separate the retrobulbar structures cleanly from the eyeball. In this way, the removal of the ciliary ganglion was assured and most of the ganglion cells that might have been situated along the course of the short ciliary nerves were excluded. Consequently, it is reasonably certain that electrical stimulation of the posterior half of the eyeball almost solely excited the postganglionic nerve fibers. Mice of strain C57 were selected because the pigmentation of the iris enables the diameter of the pupil to be observed and measured. The eyeballs were mounted in appropriately sized sockets in the base of a Lucite chamber designed to fit the mechanical stage of a microscope. The chamber contained 20 ml of Krebs-Ringer solution, buffered with bicarbonate and bubbled with a finely dispersed mixture of 95-percent O_2 and 5-percent CO_2, which maintained the *p*H at 7.4. The temperature was kept at 37°C by means of a heating lamp controlled by a mercury-in-glass thermoregulator dipped into the chamber.

The eyeball holder was a modified form of the previous design, each socket being provided with two silver electrodes, one in the form of a button touching the most posterior part of the eyeball, the other a ring in contact with the equator of the eyeball (Figs. 1 and 2). These electrodes were connected, through terminals on the outside of the chamber, to a square wave stimulator.

After a 30-minute stabilization period, pupillary diameter was determined with a microscope containing a micrometer disk in the ocular. The measurement was repeated after electrical stimulation, or after replacement of the plain Krebs solution with fresh Krebs solution containing a drug, or after both. The "response" was the ratio of the diameter at the end of a period of

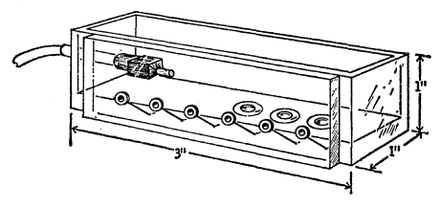

Fig. 1. Diagram of Lucite chamber. The gas was dispersed by passing it through a section of swab stick inserted in the drilled-out hub of a hypodermic needle.