Direct Injection of the Thymus with Antigenic Substances.* (29059)

JOSEPH D. SHERMAN, MARVIN M. ADNER AND WILLIAM DAMESHEK
Blood Research Laboratory, Pratt Clinic-New England Center Hospital and Department of Medicine, Tufts University School of Medicine, Boston, Mass.

The intimate relationship of the thymus to the lymphoid tissues and to the immune response has been demonstrated in a variety of animal species. This relationship is probably mediated by the lymphocyte distributing function of the gland and perhaps by humoral mechanisms as well. The thymus has been thought to provide a highly specialized (sheltered) environment in which differentiation and multiplication of cells concerned with immunity can take place without relation to antigenic stimulation(1). By virtue of its location, together with the absence of sinusoids and of afferent lymphatics and by a presumed barrier(3), the thymus seems to be "protected" from the entry of exogenous or even endogenous antigen. Although removal of a gland is an excellent way of studying its function, it is, by no means, the only method. Thus, we decided to investigate the effects induced by injection of antigen or other material directly into the thymus. Marshall and White have previously noted antibody formation, plasma cells and lymphoid follicles in the thymus of guinea pigs following direct thymic injection(4). In this study, we present a preliminary report of the first group of experiments dealing with some of the morphologic, hematologic and immunologic effects of direct injection of a variety of agents into the thymus gland.

Materials and methods. Golden hamsters *(Mesocricetus auratus)* from our stock colony, of both sexes, 2 weeks and 8 weeks of age at start of experiment, fed Purina laboratory chow and water *ad lib* were used. The animals were divided into groups based on the kind of material injected. Each substance was injected directly into the thymus, the spleen or subcutaneously. The various agents injected included: saline, bovine serum albumin (BSA, Armour), complete Freund's adjuvant (Difco), thrice washed one percent saline suspension of homologous red blood cells, saline suspension of whole homologous blood, autoclaved 5% saline suspension of activated charcoal, human gamma globulin (Fraction II, Pentex Corp., Kankakee, Ill., 36 μg of protein per 0.2 ml of saline) and homologous saline suspension of muscle. The muscle suspension was prepared from the quadriceps and hamstring muscle groups of a healthy adult hamster killed by an overdose of Nembutal (pentobarbital sodium, Abbott). Sterile instruments and equipment were used. The muscle was finely minced on a watch glass and moistened with 0.5 ml of sterile saline. The minced muscle was then gently pressed through a 30-mesh tantalum gauze into a 10 ml beaker and all adherent fragments were washed through the gauze with 4-5 cc of saline; recipient animals were injected immediately.

The same quantity of material (0.02 ml) was injected, regardless of the substance used and its route of injection. The thymus was exposed by a previously described technique (5). In the first series of experiments, one lobe of the gland was injected and a single injection was made. The spleen was exposed by a previously described method(6) and a single intrasplenic injection was made. The subcutaneous tissues of the back were injected once. A 27-gauge needle facilitated the injections which, in the case of the thymus gland and spleen, were performed under direct microscopic vision; all obvious leakage was immediately removed. A separate, sterile needle was used for each injection. In a second experimental group, whole homologous blood was injected weekly, directly into one lobe of the thymus gland, the spleen or subcutaneously. In addition, some hamsters had sham thymic injections at weekly intervals. In all cases, the same quantity of whole blood was injected (0.01-0.02 cc) using a 27-gauge needle. Blood was obtained by cardiac punc-

* Aided with funds from U.S.P.H.S. grants.

TABLE I. Summary of Experimental Groups, Material and Route of Injection.

	Age	Material	Intrathymic	Intrasplenic	Subcutaneous
Single injection	8 weeks	Saline	+	+	+
		BSA	+	—	+
		Freund's	+	+	+
		Charcoal	+	+	+
		RBC's	+	+	+
		Muscle	+	+	+
	2 "	HGG	+	+	+
Multiple injections	8 "	Whole blood	+	+	+

ture, the same hamster being used as the donor for this study. Table I summarizes the various experimental groups, materials injected and routes of administration.

At varying intervals (24 hours, 3 days, 1, 4 and 10 weeks) following injection, the animals were killed by decapitation. Hematologic tests, including total and differential WBC's, reticulocyte counts and hemoglobins were performed. Coombs' antiglobulin tests (7) and serum electrophoresis studies were performed. When indicated, sera were studied for specific anti-human gamma globulin (HGG) antibodies by a microtechnique (modified Takatsky) adapted for hemagglutination(8) and for total gamma globulin (9). Complete autopsies were performed on all animals and all tissues were studied microscopically after fixation in 10% neutral formalin and staining with hematoxylin and eosin. Selected tissues were stained with methyl green pyronine.

Results. Hematologic data. All of the hamsters had normal total and differential white cell values before injection. The normal hamster hemoglobin for the ages studied was 16.1 $\pm$ 1.3 g. Anemia (hemoglobin of $<$12.0 g/100 ml of blood) was found only in those animals in which antigenic materials were injected directly into the thymus and did not develop following either intrasplenic or subcutaneous injection. Thus, 5/15 hamsters injected intrathymically with muscle, 3/8 with red blood cells, 6/8 with Freund's adjuvant and 1/10 with charcoal became anemic. The hamsters injected intrathymically with either saline, BSA, or HGG failed to develop anemia. Multiple injections of whole hamster blood produced anemia in 6/9 hamsters injected intrathymically and failed to produce anemia in animals repeatedly injected subcutaneously or intrasplenically.

Immunologic data. Negative Coombs' antiglobulin tests were found routinely in the normal hamster. Similarly, negative tests were obtained in all hamsters injected either singly or repeatedly by intrasplenic or subcutaneous routes. However, the test became positive in a number of hamsters injected intrathymically. Thus, after one injection the test was positive with HGG in 1/13, saline 1/4, BSA 2/5, muscle 6/15, red blood cells 2/8, Freund's adjuvant 3/8 and charcoal 2/10 and after multiple injections of whole blood in 7/10. Positive tests persisted for as long as 4 weeks following a single intrathymic injection.

The normal hamster gamma globulin value for animals 2 and 8 weeks of age ranged between 0.9-1.1 g%. Normal values were found in all hamsters injected with the various materials regardless of the route injected, save in 5/15 hamsters injected intrathymically with HGG. These hamsters showed somewhat increased globulins with values ranging from 1.5-1.7 g%. Antibody titers following injection of HGG into the thymus, spleen or subcutaneous tissues were the same regardless of route of antigen injection.

Pathologic data. A single injection of material into one lobe of the thymus gland resulted in enlargement of the injected lobe and no gross changes in the non-injected lobe. This effect was observed with muscle, erythrocytes and BSA. On the other hand, Freund's adjuvant injected into one lobe of the thymus resulted in bilateral thymus lobe enlargement (Fig. 1). In addition to these

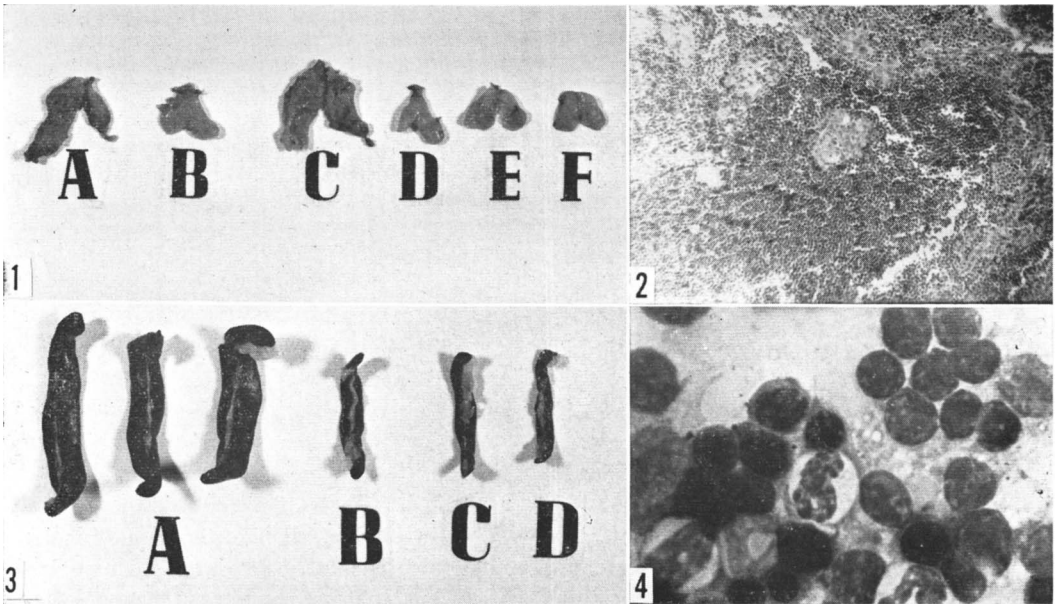

FIG. 1. Hamster thymus glands removed 2 weeks after direct injection. A, following homologous muscle; B and D, following homologous red blood cells; C, following Freund's adjuvant; and E and F, following saline. Only one lobe injected on one occasion; note that both lobes enlarged following Freund's adjuvant injection.

FIG. 2. Hamster thymus gland 30 days after direct injection of homologous red blood cells showing a medullary germinal follicle and germinal center.

FIG. 3. Hamster spleens removed 2 weeks after direct thymic injection. A, following homologous muscle; B and C, following homologous red blood cells; and D, following saline (normal size). Splenomegaly can be seen in A, B and C.

FIG. 4. Hamster bone marrow obtained from an anemic animal that was injected intrathymically with whole blood. Marrow shows erythroid and lymphoid hyperplasia.

gross observations, microscopic study of the injected and non-injected thymic lobes revealed different morphological patterns. One week following injection of Freund's adjuvant, muscle, BSA and erythrocytes, the injected thymus lobe revealed cortical hyperplasia, increase in Hassall's corpuscle activity and marked reticuloendothelial activity, scattered polymorphonuclear cells and very rare plasma cells. The cellular pattern of the non-injected lobe was comparatively unremarkable, save when Freund's adjuvant was used when both lobes showed morphologically similar modifications. On the 30th day following direct thymus gland injection, plasma cells and germinal follicles appeared in the lobe injected with Freund's adjuvant, HGG, skeletal muscle and whole blood (Fig. 2). The charcoal remained in the injected lobe and generally in a subcapsular position.

Splenomegaly was noted in 4 of 5 hamsters that were injected intrathymically with one injection of muscle (Fig. 3) and in 5 of 10 animals that were injected intrathymically with multiple injections of whole blood. The spleens weighed 428 to 750 mg (in contrast with the normal spleen weight at this age of 100-130 mg). Microscopically, the enlarged spleens showed enlarged, active Malpighian corpuscles and marked reticuloendothelial hyperplasia; immature plasma cells were noted along the trabeculae. Slight splenic enlargement was found after RBC injections into the thymus, but this change was not noted with other antigens.

Injection of Freund's adjuvant into one lobe of the thymus was followed by lesions in the lung parenchyma and subpleurally in the epicardium, liver, mesenteric nodes and ileum. The lesions consisted of focal granulomas characterized by reticuloendothelial hyperplasia, changes previously described (25-29). These changes were not produced by subcutaneous or intrasplenic injections of

a comparable amount of adjuvant.

Injection of saline, BSA or homologous red blood cells into the thymus occasionally resulted in hypertrophy of mesenteric lymph nodes, Peyer's patches and Malpighian corpuscles. These changes were of a mild degree compared with the profound changes observed following intrathymic injection of muscle, Freund's adjuvant or whole blood.

The bone marrow of the hamsters injected intrathymically with one injection of muscle or Freund's adjuvant showed slight to marked erythroid and lymphoid hyperplasia (Fig. 4) and similar changes were noted in animals multiply injected with whole blood. These changes could be correlated, in some cases, with polychromatophilia, nucleated red blood cells and spherocytes in the peripheral smear, positive Coombs' test (EAR) and splenomegaly.

Comment. Although these data are of a preliminary nature, they appear to be sufficiently important to warrant this report. Injection into the thymus of a variety of agents, some of them antigenic, resulted in hematologic, immunologic and morphologic changes that were in many respects in direct contrast with those observed following extirpation of the gland. Thus, an increase in spleen and lymph node size was noted together with bone marrow lymphocytosis. Anemia and a positive Coombs' antiglobulin test were also commonly found, together with increased levels of gamma globulin in some animals. From the morphologic standpoint, each lobe of the thymus appeared to function independently(10). The striking development of germinal follicles and plasmocytosis in the thymus following direct injection of some antigens and not of others is difficult to explain but may be related to an inability of the lymphoid cells of the gland to respond to some antigens. The various findings observed of the effects of direct injection of antigen into the thymus, *i.e.*, enlargement of lymphoid organs, increase of bone marrow lymphocytes, increase of gamma globulin, positive Coombs' test, increase in plasma cells, development of germinal follicles in the thymus, lymph nodes and spleen, might all be construed as indicating "stimulation" of the gland. The anemia, often associated with a positive Coombs' test, might be due either to non-specific coating of erythrocytes by material released from the thymus (gamma globulin) or to the presence of active erythrocyte autoantibody with subsequent increased red cell destruction. This problem is being investigated

The thymus, in contrast to other lymphoid organs, has been found to produce either no antibody or less antibody(11-15,23,24) following stimulation by a variety of antigens injected by the usual systemic routes. Several reports have indicated that the thymus ordinarily lacks plasma cells. It has been suggested that the presence of these cells together with lymphoid follicles indicates the participation of the thymus in an immunological process(2,16,22). Thus, plasma cells and lymphoid follicles have been noted in the human thymus gland in myasthenia gravis (17) and in systemic lupus erythematosus (18). Germinal follicles also occur in the thymus glands of the NZB strain of mice with genetically determined autoimmune disease(16). The same thymic lesion has been produced by injecting large doses of Freund's adjuvant subcutaneously into rats(19). It is possible that Freund's adjuvant may, in some manner, break the "thymic barrier" when injected systemically and this may account for its role in production of such experimental diseases as allergic encephalomyelitis(20) or thyroiditis(21). The possibility exists that the presumably uncommitted but potentially immunocompetent cells of the thymus could respond to direct contact with antigen with resultant production of immunological changes and even autoimmune disease. It is conceivable that the thymic lymphocytes, which are endodermally derived, might respond to antigen in a manner physiologically different from the mesodermally derived lymphocytes of the other lymphoid organs.

Summary. Direct injection of the adult hamster thymus gland with a variety of substances has produced changes in lymphoid tissues, bone marrow and blood that suggest thymic stimulation. The changes produced

by direct injection of the thymus gland include splenomegaly and lymphadenopathy, increase of bone marrow lymphocytes, increase of gamma globulin, positive Coombs' antiglobulin test, development of follicles within the thymus gland, plasma cell development in the thymus, spleen and lymph nodes and anemia, possibly of the autoimmune type.

1. Loutit, J. F., *Lancet,* 1962, v2, 1106.
2. Simpson, J. A., *Scot. Med. J.,* 1960, v5, 419.
3. Clark, S. L., Jr., *Am. J. Anat.,* 1963, v112, 1.
4. Marshall, A. H. E., White, R. G., *Brit. J. Exp. Path.,* 1961, v42, 379.
5. Sherman, J. D., Adner, M. M., Dameshek, W., *Blood,* 1963, v22, 252.
6. ———, *ibid.,* 1964, v23, in press.
7. Betts, A., Rigby, P. G., Emerson, C. P., Friedell, G. H., *J. Lab. and Clin. Med.,* 1961, v58, 652.
8. Sever, J. L., *J. Immunol.,* 1962, v88, 320.
9. Aull, J. C., McCord, J., *J. Lab. and Clin. Med.,* 1955, v4, 46.
10. Siegler, R., *The Role of the Thymus in Immunobiology,* 1963, Paul B. Hoeber, Inc., New York, in press.
11. Bjørneboe, M., Gormsen, H., Lundquist, F. R., *J. Immunol.,* 1947, v55, 121.
12. Harris, T. N., Rhoads, J., Stokes, J., Jr., *ibid.,* 1948, v58, 27.
13. Stoner, R. D., Hale, W. M., *ibid.,* 1955, v75, 203.
14. Rosenberg, L. T., Chandler, M. H., Gordon, A. S., Fischel, E. E., *ibid.,* 1958, v81, 136.
15. Askonas, B. A., White, R. G., *Brit. J. Exp. Path.,* 1956, v37, 61.
16. Burnet, F. M., *Australasian Ann. Med.,* 1962, v11, 79.
17. Castleman, B., Norris, E. H., *Medicine,* 1949, v28, 27.
18. Burnet, F. M., *The Role of the Thymus in Immunobiology,* Paul B. Hoeber, Inc., New York, 1963, in press.
19. Svet-Moldavsky, G. J., Raffkina, L. I., *Nature,* 1963, v197, 52.
20. Waksman, B. H., *J. Infect. Dis.,* 1956, v99, 258.
21. Witebsky, E., Rose, N. R., Shulman, S., *J. Immunol.,* 1955, v75, 269.
22. van der Geld, H., Feltkamp, T. E. W., van Loghem, J. J., Oosterhuis, H. J. G. H., Biemond, A., *Lancet,* 1963, v2, 373.
23. Thorbecke, G. J., Keuning, F. J., *J. Immunol.,* 1957, v78, 56.
24. Dixon, F. J., Weigle, W. O., Roberts, J. C., *ibid.,* 1957, v78, 56.
25. Suter, E., White, R. G., *Am. Rev. Tuberc.,* 1954, v70, 793.
26. White, R. G., Coons, A. H., Connolly, J. M., *J. Exp. Med.,* 1955, v102, 83.
27. Steiner, J. W., Langer, B., Schatz, D. L., *Arch. Path.,* 1960, v70, 424.
28. Schoenberg, M. D., Moore, R. D., *ibid.,* 1961, v72, 446.
29. Rupp, J. C., Moore, R. D., Schoenberg, M. D., *ibid.,* 1960, v70, 43.

Received December 31, 1963. P.S.E.B.M., 1964, v115.

In vitro Transformation of Hamster Kidney Cells by Human Adenovirus Type 12.* (29060)

WILLIAM DAVID MCBRIDE AND ANNA WIENER (Introduced by Edwin H. Lennette)
Viral and Rickettsial Disease Laboratory, California State Department of Public Health, Berkeley

The discovery of the oncogenic potential for Syrian hamsters of adenovirus type 12 provided the first direct evidence that a virus of human origin can induce cancer(1). This observation has been confirmed and extended to include adenovirus type 18(2). More recently it has been shown that cell cultures can be established *in vitro* from the virus-induced tumors(3,4). Unfortunately, a detailed analysis of an oncogenic virus-host cell relationship is exceedingly difficult if dependence must be placed on tumorigenesis *in vivo* as an experimental model, with or without subsequent *in vitro* cultivation of the tumor cells.

This report describes the transformation of hamster cells *in vitro* by adenovirus type

* The work on which these observations are based was supported by a grant from Nat. Cancer Inst., Nat. Inst. Health, U. S. P. H. S., to the California Cancer Field Research Program, Calif. State Dept. of Public Health.