redecantation. The slices were then resuspended, and uptake of DA was measured. In two experiments, the unprotected samples gave T/M ratios of only 6 and 8 percent of control values, while the catalase-protected samples gave ratios of 45 and 52 percent of control values. Triplicate samples agreed to within ± 5 percent of their mean; the T/M ratio for control tissue was 46 : 1. Since 6-OHDA accumulates in catecholamine nerve terminals (*2*), the inhibitory effects, not protected by catalase, can be attributed in part to the action of $H_2O_2$ generated within the tissue and in part to the direct action of residual 6-OHDA. Tissue slice experiments performed with [$^3$H]-5-HT produced results similar to those in Table 1, that is, the system was fully protected by catalase in the medium.

We considered the possibility that oxidation of [$^3$H]catecholamines by the quinone or other oxidation products of 6-OHDA, or by $H_2O_2$ generated within the tissue, might account for the lowered T/M ratios. In experiments with homogenates, we analyzed both the tissue and the supernatant by thin-layer chromatography (*8*) and found that more than 90 percent of the radioactivity was present as unchanged [$^3$H]catecholamine. Similar observations were made for uptake measurements in the presence of $10^{-3}M$ $H_2O_2$. Therefore, diminished uptake cannot be attributed to destruction of [$^3$H]catecholamines.

Certain enzymes such as monoamine oxidase generate small amounts of $H_2O_2$ within neural tissue. However, the mechanisms for detoxifying $H_2O_2$ are unclear. Protective enzymes, such as glutathione peroxidase or catalase, which act in other tissues to destroy relatively low or high concentrations of $H_2O_2$, respectively (*9*), appear to be very low or absent in brain (*10*). An enzyme that utilizes $H_2O_2$ to convert tyrosine to dopa has been found in human brain (*11*); perhaps this enzyme serves in a protective role. However, brain tissue is uniquely sensitive to oxidative inhibition of glycolysis when $H_2O_2$-generating agents such as the *o*- or *p*-dihydroxy phenols are added in vitro (*10, 12*). This sensitivity may contribute to the susceptibility of catecholamine nerve terminals to degeneration when 6-OHDA is injected in experimental animals.

We have shown that 6-OHDA generates $H_2O_2$ and that $H_2O_2$ can damage the uptake systems for the biogenic

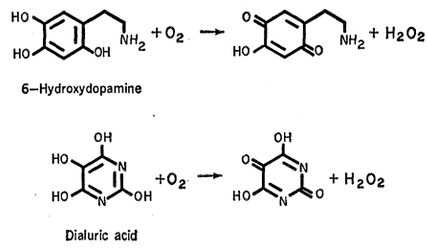


Fig. 1. Formation of hydrogen peroxide from 6-hydroxydopamine or dialuric acid by autoxidation.

amines. The formation of $H_2O_2$ from 6-OHDA has been reported by others (*13*). While release of catecholamines by 6-OHDA or competitive inhibition of uptake may be the mechanisms for the reversible loss of catecholamines in vivo, damage by $H_2O_2$ generated from the 6-OHDA that accumulates in catecholamine terminals may be the cause of the long-lasting catecholamine depletion that accompanies the destruction of nerve terminals.

RICHARD HEIKKILA
GERALD COHEN

*Columbia University,*
*College of Physicians & Surgeons,*
*640 West 168 Street,*
*New York 10032*

### References and Notes

1. K. D. Evetts, N. J. Uretsky, L. L. Iversen, S. D. Iversen, *Nature* **225**, 961 (1970); N. J. Uretsky and L. L. Iversen, *J. Neurochem.* **17**, 269 (1970); H. Thoenen and J. P. Tranzer, *Arch. Pharmak. Exp. Pathol.* **261**, 271 (1968).
2. G. Jonsson and C. Sachs, *Eur. J. Pharmacol.* **9**, 141 (1970); L. L. Iversen, *ibid.* **10**, 408 (1970).
3. F. E. Bloom, S. Algeri, A. Gropetti, A. Revuelta, E. Costa, *Science* **166**, 1284 (1969); G. R. Breese and T. D. Traylor, *J. Pharmacol. Exp. Ther.* **174**, 413 (1970).
4. D. G. Cornwell and D. W. Deamer, unpublished observations.
5. S. H. Snyder and J. T. Coyle, *J. Pharmacol. Exp. Ther.* **165**, 78 (1969). A crude pellet of synaptosomes and mitochondria was isolated by centrifugation at 27,500*g* for 30 minutes. The pellet was suspended in 10 ml of Krebs-Ringer phosphate containing glucose (1 mg/ml), Pargyline (0.016 mg/ml), and ethylenediaminetetraacetic acid (0.05 mg/ml), but no ascorbate. Portions (0.5 ml) were added to 9.5 ml of the same medium for uptake studies. Ascorbate was not added to the medium lest it interfere with the experiments since it is itself an $H_2O_2$-generating agent [B. Chance, *Biochem. J.* **46**, 387 (1950)], and a catalyst for the conversion of catalase to the inactive complex II [D. Keilin and P. Nicholls, *Biochim. Biophys. Acta* **29**, 302 (1958)]. In other experiments, ascorbate potentiated the inhibition of uptake caused by dialuric acid; this may have been due either to reduction of alloxan to dialuric acid or to the $H_2O_2$ generated by the ascorbate.
6. The crystalline catalase suspension (1 ml) was centrifuged at 700*g* for 10 minutes. The supernatant was discarded, and the crystals were dissolved in 10 ml of Krebs-Ringer phosphate solution. Samples of 0.5 ml were used.
7. Aminotriazole was added to 4 ml of the catalase solution (*6*) to give a concentration of 0.1*M*, and the catalase was inhibited by exposing the samples to $H_2O_2$ vapor for 2 hours at 37°C with a gaseous diffusion method in Warburg vessels [G. Cohen and P. Hochstein, *Science* **134**, 1756 (1961); *Biochemistry* **3**, 895 (1964)]. Aminotriazole by itself had no effect in the uptake system.
8. G. Cohen and M. Collins, *Science* **167**, 1749 (1970).
9. G. Cohen and P. Hochstein, *Biochemistry* **2**, 1420 (1963); L. Flohé and I. Brand, *Biochim. Biophys. Acta* **191**, 541 (1969).
10. G. Cohen and P. Hochstein, *Dis. Nerv. Syst.* **24** (Suppl.), 44 (1963).
11. L. Edelstein, M. Okun, N. Or, *Abstr. Div. Biol. Chem. Amer. Chem. Soc. Nat. Mtg. 160th* (1970), p. 192.
12. P. Hochstein and G. Cohen, *J. Neurochem.* **5**, 370 (1960).
13. Unpublished observations of F. Bigler as quoted by H. Thoenen, in *New Aspects of Storage and Release Mechanisms of Catecholamines*, H. Schumann and G. Kroneberg, Eds. (Springer-Verlag, New York, 1970), p. 130.
14. We thank Dorothy Dembiec and Felicitas Cabbat for assistance. Supported by PHS grants NS-05184 and MH-17071.

25 January 1971

## Immunocompetent Cells among Mouse Thymocytes: A Minor Population

Abstract. *Suspensions prepared from thymuses of TL+ mice contain a majority of TL+ cells and a minority of TL− cells. The graft versus host reactivity of the TL− population is much greater than that of the whole population, as judged by the numbers of cells required to give splenic enlargements in Simonsen's assay. It is proposed that the TL− thymocyte represents a stage in the differentiation of TL+ thymocytes into immunocompetent lymphocytes.*

Thymocytes can cause graft versus host reactions (GVHR) but their capacity to do so is greatly inferior to that of lymph node cells, many more thymocytes than lymph node cells being required to produce GVHR (*1*). Indeed it is necessary to consider the possibility that the low GVH activity of suspensions of thymocytes may in fact be due to contamination with immunocompetent cells from the blood or from lymph nodes unintentionally included when thymocyte suspensions are prepared (see below). If such contamination can be ruled out, then two explanations may be suggested for the low GVH activity of thymocyte suspensions—either the activity of individual thymocytes is lower than that of individual lymph node cells, or the

proportion of thymocytes that can generate GVHR is less than that of lymph node cells. In the latter case it can be postulated that there is a subpopulation of thymocytes whose GVH activity approaches that of lymphoid cells and which accounts for the entire GVH capacity of thymocyte suspensions.

In the experiments reported here we made use of the thymocyte-specific antigen TL (*2, 3*) to investigate these two possibilities. All cell suspensions were prepared from the thymus, lymph nodes, or blood of either C57BL/6 mice, whose thymocytes do not carry TL antigens (*abbr* C57/TL$^-$), or from mice of the congenic stock C57/TL$^+$ which differs from C57BL/6 at the *Tla* locus, the thymocytes of this strain carrying antigens TL.1,2, and 3 (*4*).

In preparing thymocyte suspensions, special care was taken to remove the parathymic lymph nodes adherent to the capsule (*5*); these can easily be made visible by injecting carbon black (*6*) intraperitoneally 30 minutes beforehand (Fig. 1). Newborn (BALB/c $\times$ C57/TL$^-$)$F_1$ mice (*abbr* $F_1$ neonates) were inoculated intravenously with cells from C57/TL$^-$ or C57/TL$^+$ female donors 2 to 3 months old (*7*). Graft versus host reactivity was determined by Simonsen's method (*8*), a mean spleen index (MSI) of 2 or more in a total of at least five mice being taken as a positive GVH test.

First, the capacity of C57/TL$^+$ and C57/TL$^-$ thymocytes to produce GVHR was compared with that of lymph node cells; $15 \times 10^6$ C57/TL$^+$ or C57/TL$^-$ thymocytes gave MSI of $2.5 \pm 0.39$ (standard deviation) and $2.3 \pm 0.42$, respectively, and $5 \times 10^6$ C57/TL$^+$ or C57/TL$^-$ thymocytes gave near normal MSI of 1.2 and 1.1, respectively. In contrast, as few as $2 \times 10^5$ C57/TL$^+$ lymph node cells gave an MSI of $2.6 \pm 0.46$ (Fig. 2). Thus it required 75 times as many thymocytes to give GVHR comparable with that of lymph node cells.

Thymocyte suspensions from TL$^+$ mice contain a minority of TL$^-$ cells. The proportion of thymocytes that survive exposure to TL antiserum and complement is affected by many circumstances (age and strain of mouse, homozygosity versus heterozygosity for *Tla*, source of complement, and doubtless other factors), and ranges from 3 to 20 percent. Thus excluding serological artifacts, the thymocytes of C57/TL$^+$ mice and of mice from other TL$^+$ strains can be divided into a major TL$^+$ population and a minor TL$^-$ population. The GVH reactivity of the latter was tested in the following manner. Suspensions containing (i) $15 \times 10^6$ C57/TL$^+$ thymocytes previously exposed to excess antiserum to TL and complement (*9*) or (ii) $15 \times 10^6$ untreated thymocytes were injected into $F_1$ newborns. The thymocytes treated with antiserum, of which 13 percent survived, gave an MSI of $2.6 \pm 0.62$, which was very similar to the index of $2.8 \pm 0.40$ for untreated thymocytes (Fig. 3). For the controls, treatment of $15 \times 10^6$ C57/TL$^-$ thy-

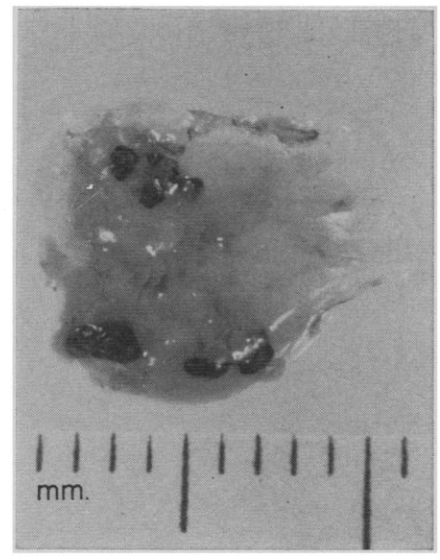


Fig. 1. Dorsal view of mouse thymus showing most of the capsule, with several adherent lymph nodes, ½ hour after intraperitoneal injection of carbon black (*6*).

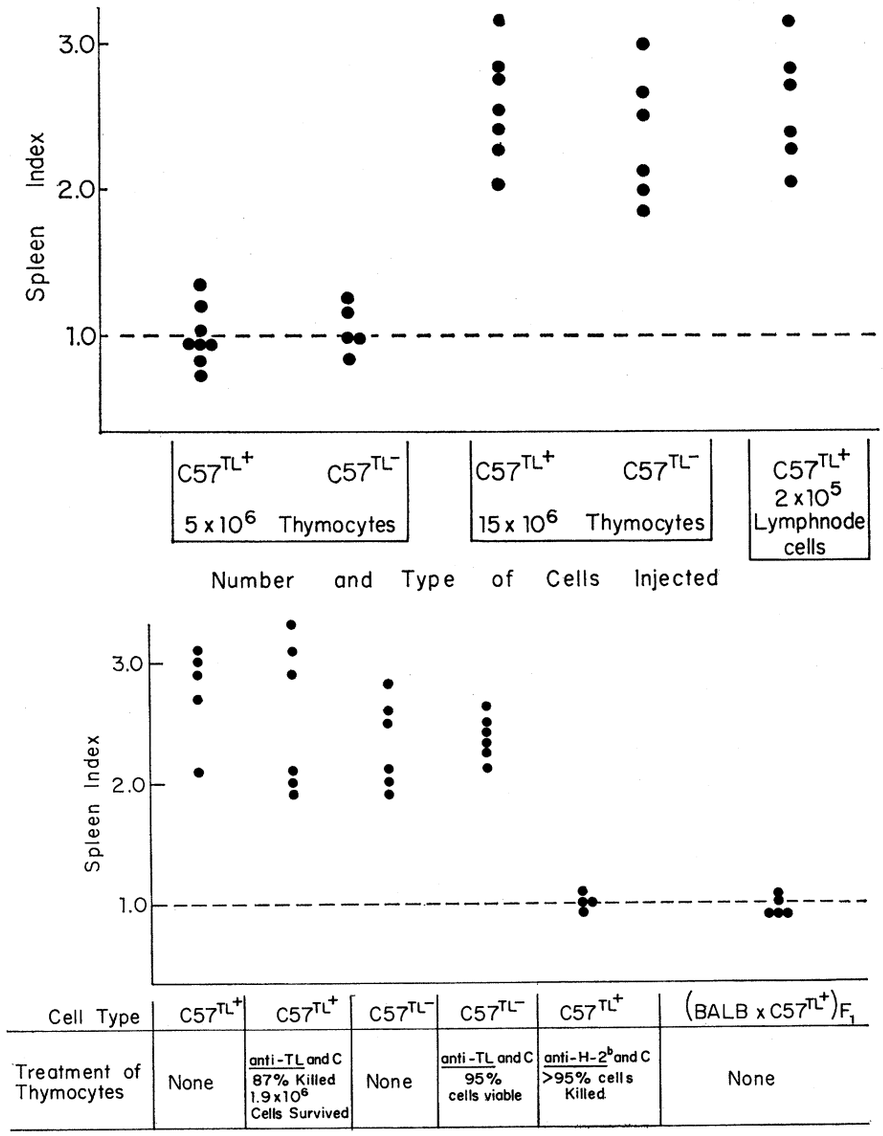


Fig. 2 (top). Comparative GVH activity of thymocytes and lymph node cells according to Simonsen assay (one dot represents one mouse). Fig. 3 (bottom). Effect of exposure to cytotoxic TL or H-2 alloantiserums and complement on GVH activity of thymocytes (Simonsen assay, one dot represents one mouse).

mocytes with TL antiserum and complement (more than 95 percent of the cells surviving) gave a similar MSI, $2.6 \pm 0.20$; treatment of C57/TL$^+$ thymocytes (H-2$^b$) with H-2$^b$ antiserum plus complement (which killed over 95 percent of them) gave no GVHR (Fig. 3). Thus the minority population of TL$^-$ cells apparently contains most of the cells capable of producing GVHR (*10*).

The following consideration excludes the possibility that this minor population consists solely of blood lymphocytes contaminating the thymocyte suspensions. In control experiments, $1 \times 10^5$ blood leukocytes did not produce splenic enlargement whereas $2 \times 10^5$ blood leukocytes gave an MSI of $1.6 \pm 0.41$. The number $2 \times 10^5$ corresponds to about 0.02 ml of whole blood; since contamination of our thymocyte suspensions with erythrocytes never approached 5 percent, it is obvious that significant contamination with leukocyte-containing blood can be ruled out.

As for the possibility of some form of selective trapping of immunocompetent circulating cells by the thymus, the following data render this unlikely—$15 \times 10^6$ C57/TL$^+$ thymocytes from neonatal donors (less than 24 hours old) gave an MSI of $2.7 \pm 0.57$ in six mice, indicating a GVH activity comparable with that of adult thymus [see also (*11*)]. But the spleens of neonatal mouse donors, and presumably their circulating blood, contain too few immunocompetent cells for demonstrable GVHR (*12*). Thus selective trapping of peripheral immunocompetent cells is scarcely a probable explanation of the GVH activity of the neonatal thymocyte population. We conclude that the immunocompetent (TL$^-$) fraction of both neonatal and adult thymocyte populations is derived in situ.

Immunoselection with cytotoxic TL antiserum has proved a feasible tool for isolating a discrete subpopulation of thymocytes, a subpopulation which carries most and possibly all of the GVH reactivity of thymus.

In normal mice, TL$^+$ cells are not found outside the thymus, which implies that peripheral migration of thymocytes entails preceding loss of TL antigens. Thus much evidence now points to the existence in thymus of a proportion of cells that have taken several maturative steps, including not only the loss of TL and the acquisition of immunocompetence but also major quantitative changes affecting surface antigens other than TL [see review (*3*)]. These maturation steps may not occur simultaneously, and this is perhaps the most likely reason why the GVH capacity of the subpopulation selected, while considerably greater than that of the unselected thymocyte population, is still only about one-tenth of that possessed by lymph node lymphocytes.

E. LECKBAND
E. A. BOYSE

*Sloan-Kettering Institute for Cancer Research, New York 10021*

### References and Notes

1. R. E. Billingham and W. K. Silvers, in *The Thymus*, V. Defendi and D. Metcalf, Eds. (Wistar Inst. Press, Philadelphia, 1960), p. 41.
2. L. J. Old, E. A. Boyse, E. Stockert, *J. Nat. Cancer Inst.* **31**, 977 (1963).
3. E. A. Boyse and L. J. Old, *Annu. Rev. Genet.* **3**, 269 (1969).
4. E. A. Boyse, E. Stockert, L. J. Old, in *International Convocation on Immunology*, N. R. Rose and F. Milgrom, Eds. (Karger, Basel, 1968), p. 353. The stock used in the present experiments was derived from the 18th generation of backcrossing to C57BL/6.
5. J. N. Blau and J. M. Gaugas, *Immunology* **14**, 763 (1968).
6. Pelikan (Gunther Wanger, C11/1431 a). The Pelikan was centrifuged at 2000 rev/min for 15 minutes. The supernatant was diluted one-half in medium 199, and 0.2 ml was injected intraperitoneally into a mouse. The mouse was killed ½ hour later by cervical dislocation, care being taken to avoid bleeding near the thymus.
7. R. E. Billingham, in *Transplantation of Tissues and Cells*, R. E. Billingham and W. K. Silvers, Eds. (Wistar Inst. Press, Philadelphia, 1961), p. 90. We used medium 199 with $NaHCO_3$ and glutamine (Grand Island Biological, Chagrin Falls, Ohio).
8. M. Simonsen, *Progr. Allergy* **6**, 349 (1962).
9. The antiserum was C57BL/6 antiserum to A strain leukemia ASL1 [see (*2*)]. The conditions of exposure to antiserum and complement were similar to those used in cytotoxic tests [E. A. Boyse, L. J. Old, I. Chouroulinkov, *Method. Med. Res.* **10**, 373 (1964)]. Washed C57/TL$^+$ thymocytes ($500 \times 10^6$), in 25 ml of medium 199, were incubated for 50 minutes at 37°C with 25 ml of TL antiserum (1:50; cytotoxic titer, 1:4000 on C57/TL$^+$ thymocytes) and 25 ml of guinea pig serum (1:4; selected for low toxicity to thymocytes). The cells were recovered by slow centrifugation, viability was checked with trypan blue, and cell concentration was adjusted to give the number of cells required for injection in 0.06 ml.
10. This conclusion is consistent with a report that systemic administration of cortisone caused the disappearance of TL$^+$ cells from thymus [M. Schlesinger and V. K. Golakai, *Science* **155**, 1114 (1967)], and an independent report that cortisone treatment combined with sublethal total-body irradiation gave rise 2 days later to a thymus cell population with tenfold increased GVH reactivity [H. Blomgren and B. Andersson, *Exp. Cell Res.* **57**, 187 (1969)].
11. G. J. Thorbecke and M. W. Cohen, in *The Thymus*, V. Defendi and D. Metcalf, Eds. (Wistar Inst. Press, Philadelphia, 1964), p. 33.
12. In this study and in previous experiments (E. Leckband and L. W. Law, unpublished), spleen cells from donors less than 24 hours old did not produce GVHR.
13. Supported in part by National Cancer Institute grant CA 08748 and a grant from the John A. Hartford Foundation, Inc.

11 December 1970

## Electrophysiological Observations in Normal and Dystrophic Chicken Muscles

Abstract. *Intracellular recordings were made on the fast posterior latissimus dorsi muscles of normal and dystrophic chickens. In the dystrophic chickens, the rate of rise of the action potential was decreased. With repetitive indirect stimulation, the action potentials decreased in size and disappeared; only an end-plate potential remained. Membrane resistance, membrane capacitance, and duration of miniature end-plate potentials were increased. A decrease in sodium permeability may be in part responsible for the observed alterations in the electrical properties of the nerve terminal and postsynaptic muscle membrane.*

Inherited muscular dystrophy in chickens was first reported in 1956 (*1*) and was found to be similar to certain forms of the disease in man (*2*). The most consistent outward sign of the disease, which primarily affects the fast, white muscles, is a progressive inability of the chicken to right itself when placed on its back (*3*). The disease symptom first appeared in our chickens (*4*) 2 to 3 weeks ex ovo, and after 6 weeks the birds could not rise because of the limited range of wing movements (*5*). Although numerous studies have revealed biochemical abnormalities in dystrophic chicken muscles (*6*), none have dealt with the electrophysiological characteristics of the motor-nerve terminal and muscle membrane. We have therefore examined the fast posterior latissimus dorsi (PLD) muscles of normal and dystrophic chickens by standard electrophysiologic techniques for recording and stimulation (*7, 8*).

Our experiments briefly describe some alterations in the pre- and postsynaptic membranes of the dystrophic neuromuscular junction and in the extrajunctional muscle membrane. In dystrophic muscle of the chicken, there is a decrease in the frequency and amplitude of spontaneous transmitter release and an increase in the time course of the individual potentials. There was a two- to threefold increase