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Thymosin Corrects the Abnormal DNA Synthetic Response of NZB Mouse Thymocytes

(autoimmune disease)

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ABSTRACT **New Zealand Black (NZB) mice develop after 16 weeks of age an autoimmune and lymphoproliferative disease which is a model for systemic lupus erythematosus and lymphoid malignancy in humans. At this age, the mice manifest a progressive decline in T lymphocyte (thymus-derived lymphocyte) functions and serum thymosin levels. Thymocytes from 8-week old NZB mice exhibit an abnormal DNA synthetic response when transplanted into lethally irradiated C57Bl/6 recipients. DNA synthesis (measured as the incorporation of radioactively labeled 5-iodo-2′-deoxyuridine) is delayed in onset and still increasing 6 days after cell transfer. By contrast, 2-week old NZB thymocytes show a normal response which is rapid in onset and completed by day 6.**

NZB mice were injected with thymosin fraction 5 or with bovine serum albumin starting at 2 weeks of age. Thymocytes from 8-week old thymosin-treated mice showed a normal DNA synthetic response, whereas the albumin-treated controls showed the abnormal response expected at this age. The ability of thymosin to correct the DNA synthetic response was related to dose and duration of treatment. These results suggest that thymosin can induce a more normal state of thymocyte differentiation in NZB mice. If abnormal thymocyte differentiation is related to the subsequent emergence of autoimmunity and lymphoid malignancy, then continuous treatment with thymosin may have therapeutic potential. These experiments suggest that an endocrine disturbance may contribute to autoimmune and lymphoproliferative disease in NZB mice and possibly in humans.

The New Zealand Black (NZB) and related hybrid mice are a useful model for the study of autoimmune and lymphoproliferative disease in man. These mice spontaneously develop findings characteristic of three human disorders, systemic lupus erythematosus, Sjogren's syndrome, and Waldenstrom's macroglobulinemia (1–4). These alterations appear after 16 weeks of age and include Coomb's positive hemolytic anemia, antibodies to nucleic acids, immune complex glomerulonephritis and monoclonal macroglobulinemia. Genetic, immunologic, and virologic factors have been implicated in the pathogenesis of their disease (3, 5).

NZB mice show a progressive loss of T-cell (thymus-derived lymphocytes) functions during their lifespan (5). Even prior to the onset of autoimmunity, their T-cells are resistant to the development of immunologic tolerance (6–8). Later in life, the mice have marked deficiencies of various T-cell effector functions such as reactivity to mitogens (9, 10) and ability to induce graft-versus-host disease (11, 12). At this stage, there is also a deficiency of long-lived recirculating T-cells (13, 14).

Dauphinee and Talal (15) have shown that the kinetics of DNA synthesis in thymocytes of 8-week old NZB mice when transferred into lethally irradiated histoincompatible recipients differ from those of control DBA/2 thymocytes. Measurement of DNA synthesis was based on the incorporation of ^{125}I-labeled deoxyuridine ([^{125}I]IdU). DBA/2 thymocytes from 2- and 8-week old donors and NZB thymocytes from 2-week old donors showed the expected DNA synthetic response upon encountering the foreign histocompatibility antigens of the recipient animals. A rapid synthetic response occurred which was maximum between days 3 to 5 and had ceased by the sixth post-transplant day. By contrast, thymocytes from 8-week old NZB mice showed an abnormal DNA synthetic response characterized by a delayed onset with peak response on day 6. These results suggested an abnormality of thymocyte differentiation, perhaps genetically determined or induced by a latent viral infection.

The present study was undertaken to determine whether thymosin, a thymic hormone, may have a role in the abnormal DNA synthetic response of NZB thymocytes. Recent studies by Goldstein and White (16) have established an important role for thymosin in the development and expression of T-cell characteristics and functions. Thymosin was first isolated from bovine thymus glands in 1966 (17) and further purified in 1972 (18). The 1972 isolation procedure has recently been modified to increase the availability of thymosin for biological and chemical studies (19). In the present report, thymosin fraction 5 (18) was used.

Bach *et al.* (20) recently demonstrated the presence of thymosin-like material in normal mouse serum and its absence in the serum of congenitally thymus-deficient ("nude") mice. This thymosin-like activity in the serum declines with age in normal mice; the decline occurs prematurely in the serum of NZB and NZB/NZW F_1 hybrid mice. By two months of age, at a time when abnormal thymocyte proliferation (15) and T-cell resistance to tolerance (6, 8) are already present, serum thymic activity is insignificant in NZB mice (20). These findings suggest that a deficiency of thymosin may contribute to the T-cell abnormalities characteristic of the NZB strain.

We have attempted to preserve a normal DNA synthetic response in the thymocytes of 8-week old NZB mice by treating the mice with various regimens of thymosin. The data

Abbreviations: T-cells, thymus-derived lymphocytes; BSA, bovine serum albumin.

obtained demonstrate that treatment with thymosin can correct the abnormal DNA proliferation pattern of NZB thymocytes.

MATERIALS AND METHODS

Mice. NZB mice were from our colony maintained at the Vivarium of the University of California, San Francisco. Eight-week old C57Bl/6 mice were purchased from Jackson Laboratory, Bar Harbor, Maine.

Irradiation Procedure. Recipient C57Bl/6 mice received 750R total body x-irradiation from a Quadacondex Westinghouse x-ray machine with a 0.5 mm of Cu and 1 mm of Al filter at 15 mA and 230 kVp. The dose rate was 122 R/min.

Preparation of Thymosin. The method of Goldstein *et al.* (18) as modified recently by Hooper *et al.* (19) was used to prepare the calf thymosin fraction 5 employed in this study. The purification procedure through fraction 5 is as follows. Thymus tissue is homogenized in 3 volumes of 0.9% NaCl with 0.5% (vol/vol) octyl alcohol added to minimize foaming. The homogenate is centrifuged at 14,000 × *g*. Three liter portions of the supernatant are heated with stirring in a boiling water bath. When the temperature reaches 80°, the sample is rapidly cooled in an ice water bath. The heat-coagulated materials are removed by centrifugation and the supernatant is added to 5 volumes of acetone that have been cooled to −10°. The precipitate that forms is collected by filtration, washed several times with cold acetone, and then dried in a vacuum desiccator under reduced pressure. The white powder is suspended in 10 volumes of 10 mM phosphate buffer (pH 7). After stirring at room temperature for 1 hr, a small amount of insoluble residue is removed by centrifugation, and the supernatant brought to 25% of saturation with a saturated $(NH_4)_2SO_4$ solution (pH 7). The precipitate is centrifuged off and the supernatant adjusted to pH 4 with 10% acetic acid. Solid $(NH_4)_2SO_4$ is added to 50% of saturation. The resulting precipitate is collected by centrifugation, dissolved in 10 mM Tris·HCl (pH 8) and subjected to ultrafiltration with the Amicon DC-2 hollow fiber system (concentration mode, HIDP 10 membrane cartridge). The ultrafiltrate is concentrated *in vacuo* by rotary evaporation at a bath temperature of 30° and desalted on a 50 × 80 cm column of Sephadex G25 equilibrated with deionized water. The protein fraction eluted prior to the salt and nucleotide peak is concentrated by rotary evaporation, as above, and lyophilized. This thymosin preparation (fraction 5) is stored desiccated at −20° and is readily soluble in phosphate-buffered saline.

Administration of Thymosin. Thymosin fraction 5 was dissolved in phosphate-buffered saline and injected intravenously into 6 groups of 15–25 NZB mice each. The total amount of thymosin administered in 3–9 injections of 0.1 ml each varied from 0.3 to 3.0 mg (Table 1). In five experiments, age and sex-matched NZB mice received the same amount of bovine serum albumin (BSA) in phosphate-buffered saline as a control protein. In a sixth experiment, untreated NZB mice served as controls.

Thymus Cell Suspensions. Thymus cells were prepared as described previously (15); 50 × 16^6 viable thymus cells were injected intravenously (0.5 ml) into each of 4–8 recipients in each experimental determination. All mice received drinking water containing 1 mg/ml of Panamycin (Upjohn, Kalamazoo, Mich.) 1 week prior to lethal irradiation and during all subsequent testing.

DNA Synthesis. The procedure of Gershon and Hencin (21) was used in which DNA synthesis is measured as uptake of ^{125}I-labeled 5-iodo-2′-deoxyuridine ([^{125}I]IdU Amersham-Searle Corp., Des Plaines, Ill., specific activity 8.4 μCi/μg). Each mouse received 2 μCi of [^{125}I]IdU intraperitoneally in 0.2 ml of saline 24 hr before spleen and femoral lymph nodes were removed. The % IdU incorporated was determined according to the expression:

$$\frac{\text{cpm(organ from thymocyte injected recipient)} - \text{cpm(organ from noninjected recipient)}}{\text{cpm(total isotope injected)} - \text{cpm (organ from noninjected recipient)}} \times 100 = \%\ [^{125}\text{I}]\text{IdU incorporation}$$

Cytotoxicity Assay. In vitro cytotoxic activity of sensitized lymphocytes was determined according to the method of Brunner (22) with slight modifications (15). Cytotoxicity was measured against ^{51}Cr-labeled EL-4 lymphoma target cells. Specific cytotoxicity was determined by measuring the release of ^{51}Cr into the medium compared to maximal release (water lysis) using the following expression:

$$\frac{\text{experimental } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}{\text{maximal } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}} \times 100 = \%\ \text{specific release}$$

RESULTS

NZB thymocytes were injected into lethally irradiated C57Bl/6 recipients. DNA synthesis by the spleen and lymph node-seeking populations was studied separately. The proliferation of spleen-seeking thymocytes from mice 2 and 8 weeks of age is shown in Fig. 1. Thymocytes from 2-week old animals responded rapidly with the peak [^{125}I]IdU incorporation occurring on day 4. Incorporation returned to baseline by day 6. Similar kinetics of DNA synthesis are seen with thymocytes from 2 and 8 week-old DBA/2 mice (15) and with other normal control strains (21). By contrast, the cells from 8-week old NZB mice show almost no response on day 4, a slight response on day 5 and a peak response on day 6. This is defined as an abnormal response.

The proliferation of lymph node-seeking thymocytes from NZB mice 2- and 8-weeks of age is also shown in Fig. 1. The kinetics of response were similar to those seen with the spleen-seeking population. The cells from the older animals again showed a delayed onset and no return to baseline.

The results with thymosin-treated NZB mice are shown in Table 1 and Figs. 2, 3 and 4. All mice were studied at 8 weeks. In group A, thymosin treatment was started at 14 days of age and maintained for a total dose of 0.9 mg given over 6 weeks. Age and sex-matched control NZB mice were untreated. At 8 weeks of age, both thymosin-treated and untreated mice were sacrificed, and thymocytes injected into irradiated C57Bl/6 recipients. As shown in Fig. 2, the kinetics of DNA synthesis were markedly different in the two groups. Thymosin-treated mice showed the response characteristic of thymo-

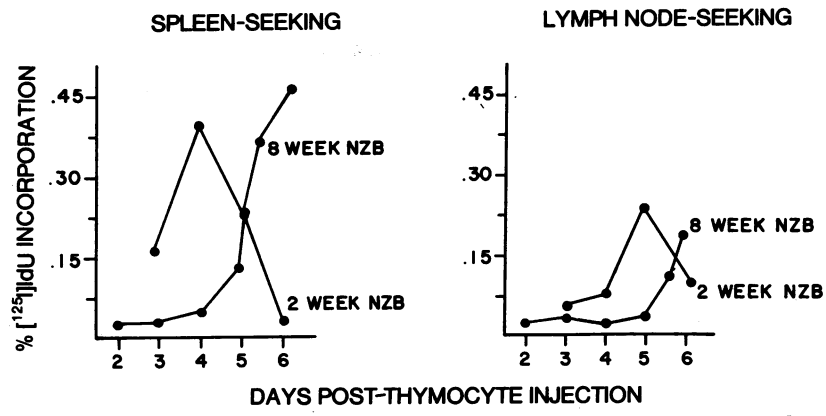

FIG. 1. Proliferation response. Incorporation of [^{125}I]IdU by spleen-seeking and lymph node-seeking thymocytes from 2- and 8-week old NZB donors injected into lethally irradiated C57B1/6 recipients.

cytes from NZB mice 2 weeks of age, whereas the untreated mice had the abnormal response expected at 8 weeks of age.

In groups B and C (Table 1 and Fig. 3), treatment was started at 21 and 28 days for a total dose of 0.7 and 0.3 mg, respectively. As a control, age and sex-matched mice received the same quantity of bovine serum albumin (BSA) injected in an identical schedule. Mice receiving thymosin showed a DNA proliferative response characteristic of 2-week old NZB thymocytes, whereas mice receiving BSA showed the customary delayed onset and abnormal response (Fig. 3).

In groups D and E (Table 1 and Fig. 4) treatment started at 35 days of age. Group D (receiving 0.3 mg of thymosin) behaved like untreated mice and showed an abnormal response. By contrast, group E (receiving 3.0 mg of thymosin) showed maintenance of the normal young response pattern. Group F, starting thymosin at 49 days and receiving only 0.3 mg of thymosin, showed no effect of treatment. Effector cell activity was assayed in this experiment; there was no difference in results between thymosin-treated and BSA-treated mice (Fig. 4).

DISCUSSION

The progressive loss of T-cell functions throughout life is now well documented in NZB mice (3, 5). Many of the more severe abnormalities occur after the onset of autoimmunity and are probably a consequence rather than cause of the disease. However, two abnormalities of thymocyte function appear before the onset of autoimmunity, i.e., at about 4 weeks of age. These two abnormalities are: (*1*) a relative resistance to immunologic tolerance (6–8), and (*2*) an abnormal proliferative response to alloantigens (15).

The basis for the T-cell abnormalities in NZB mice is unknown. Profitt, Hirsch, and Black (23) have recently shown that thymocytes infected with Moloney leukemia virus become cytotoxic for noninfected syngeneic cells. NZB thymocytes are infected with murine leukemia virus, readily demonstrable by electron microscopy as C-type particles (24, 25). If such viral infected thymocytes become cytotoxic for other noninfected thymocytes, a progressive loss of T-cell activity might develop.

NZB mice produce, early in life, an autoantibody capable of destroying thymocytes from NZB and other mouse strains (26). A thymocytotoxic antibody of this type might also contribute to a state of T-cell deficiency.

Structural (27) and functional (20) studies suggest thymic hormone deficiency as another potential cause of thymic and T-cell hypofunction. The loss of serum thymic activity in NZB mice correlates in time with the abnormalities of thymic tolerance and proliferation. This suggests that a thymic hormone deficiency may lead to the abnormal state of thymocyte differentiation. Our results support this hypothesis and indicate that abnormal thymocyte differentiation can be prevented by administration of thymosin either prophylactically to young mice (under 28 days) or in larger doses to older mice (35 days). The working hypotheses are that the abnormal thymocyte proliferation will correlate with the subsequent emergence of autoimmune disease and that corrective treatment with thymosin may have therapeutic possibilities.

Substantial evidence now exists for the importance of thymic humoral factors. These factors probably act locally in the thymus and also on T-cells in peripheral lymphoid organs. The fact that thymocyte function itself is affected in NZB mice suggests a local action of thymosin. An additional peripheral action is a distinct possibility, particularly since thymic activity is present in normal serum (20).

The mechanism by which the endocrine function of the thymus induces the differentiation and maturation of T-cells

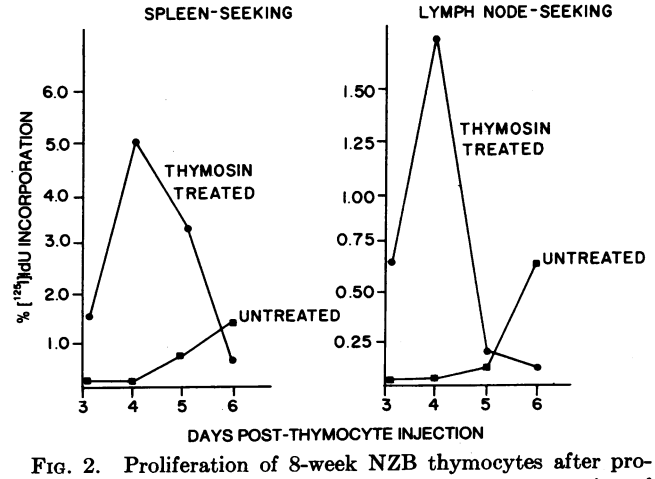

FIG. 2. Proliferation of 8-week NZB thymocytes after prolonged *in vivo* thymosin treatment for 6 weeks. Incorporation of [^{125}I]IdU by spleen-seeking and lymph node-seeking thymocytes from thymosin-treated and untreated NZB donors. Treatment was started at 14 days; each thymosin-treated mouse received an intravenous injection of 0.1 mg of thymosin in 9 injections at intervals of 5 days.

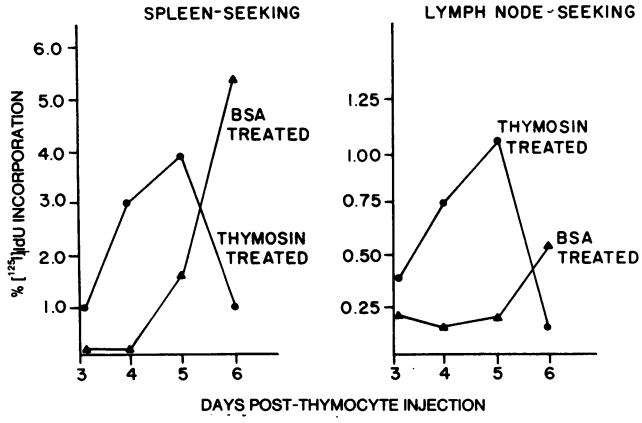

FIG. 3. Proliferation of 8-week NZB thymocytes after prolonged *in vivo* thymosin-treatment for 4 weeks. Incorporation of [^{122}I]IdU by spleen-seeking and lymph node-seeking thymocytes from thymosin-treated or albumin (BSA)-treated NZB donors. Treatment was started at 28 days; each mouse received 3 weekly intravenous injections of either 0.1 mg of thymosin or bovine serum albumin in 0.1 ml.

remains to be established. It is evident that in addition to acting *in situ*, the thymus gland produces thymosin and perhaps other hormones and/or factors which can act peripherally to induce differentiation of T-cells. Recent studies indicate that thymosin acts to activate or derepress a stem cell (predetermined T-cell?) that has all of the capacity to function as a mature T-cell. The last step in this activation process occurs very rapidly (28–30).

Autoantibodies such as rheumatoid and antinuclear factors appear spontaneously in older people. There is evidence that B-lymphocytes (bone marrow-derived lymphocytes) with receptors for autoantigens are present in normal individuals, even in the absence of serum autoantibodies. These observations suggest that a predisposition to autoimmunity may be common, and that a normal control mechanism exists to

TABLE 1. *Proliferation of thymocytes of 8-week old NZB mice after thymosin treatment*

Group	Age at beginning of treatment (days)	No. of mice	No. of intravenous injections	Total thymosin (mg)	Proliferative response*
A	14	9	9	0.9	Normal
B	21	9	7	0.7	Normal
C	28	12	3	0.3	Normal
D	35	10	3	0.3	Abnormal
E	35	8	3	3.0	Normal
F	49	16	3	0.3	Abnormal

* Relative to uninjected or BSA-treated controls.

Group A received 0.1 mg of thymosin every 5 days.

Group B received either 0.1 mg of thymosin or BSA every 5 days.

Groups C, D received weekly injections of either 0.1 mg of thymosin or BSA in 0.1 ml.

Group E received 3 weekly injections of 1.0 mg of thymosin or BSA in 0.1 ml.

Group F received 3 injections of either 0.1 mg of thymosin or BSA in 0.1 ml every other day 1 week before assay.

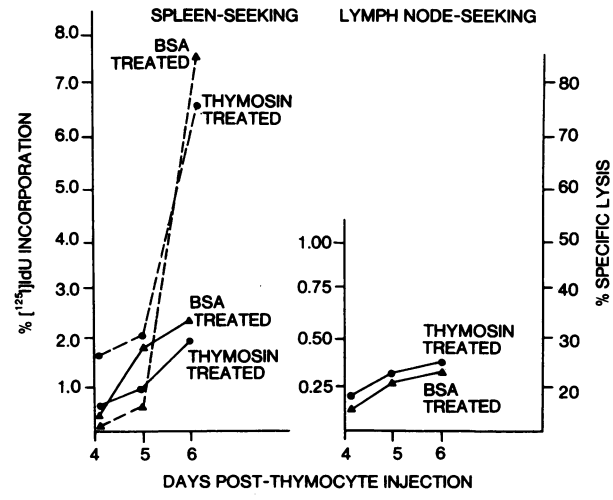

FIG. 4. Proliferation and effector response of 8-week NZB thymocytes after 1 week of thymosin or BSA. DNA synthetic (% IdU incorporation) and effector cell (% specific lysis) responses of spleen and lymph node-seeking thymocytes from thymosin-treated or albumin (BSA)-treated NZB donors. Treatment was started at 49 days; each mouse received 3 intravenous injections of either 0.1 mg thymosin or BSA in 0.1 ml every other day one week before assay. ——, proliferation response; – – –, effector response; ●, thymosin-treated; ▲, BSA-treated.

prevent selectively autoantibody production. A control of this type could be attenuated as a consequence of aging and it may be of relevance that serum thymosin levels are greatly decreased after the age of 35 or 40 (31).

A prominent role in this control mechanism probably can be assigned to the thymus. Thymectomy of normal mice leads to the premature development of Coomb's positive and antinuclear factors. Several authors have suggested that the thymus may express this control through a regulatory mechanism dependent upon suppressor factors or suppressor T-cells (5, 32, 33). This suppression would act to prevent autoantibody production by B-cells and its effectiveness might diminish with age. Our results suggest that suppression of autoimmunity may be dependent upon thymosin and perhaps other thymic humoral factors. Thymosin could act either to induce the appearance of new T-cells with suppressor-like characteristics or to influence aberrant T-cells to revert to more normal modes of function. These results raise the interesting possibility that an endocrine disturbance may underlie many of the immunologic abnormalities associated with autoimmunity and lymphoid malignancy in New Zealand mice and, by analogy, in humans.

1. Howie, J. B. & Helyer, B. J. (1968) *Advan. Immunol.* **9**, 215–266.
2. Mellors, R. C. (1965) *Int. Rev. Exp. Pathol.* **5**, 217–252.
3. Talal, N. (1974) *Progress in Clinical Immunology* (Grune and Stratton, New York), Vol. 2, in press.
4. Sugai, S., Pillarisetty, R. J. & Talal, N. (1973) *J. Exp. Med.* **138**, 989–1002.
5. Talal, N. (1970) *Arthritis Rheum.* **13**, 887–894.
6. Playfair, J. H. L. (1971) *Immunology* **21**, 1037.
7. Staples, P. J., Steinberg, A. D. & Talal, N. (1970) *J. Exp. Med.* **131**, 123.
8. Jacobs, M. E., Gordon, J. K. & Talal, N. (1971) *J. Immunol.* **197**, 359.
9. Leventhal, B. G. & Talal, N. (1970) *J. Immunol.* **194**, 918–923.

10. Rodey, G. E., Good, R. A. & Yunis, E. J. (1971) *Clin. Exp. Immunol.* **9**, 305–311.
11. Cantor, H., Asofsky, R. & Talal, N. (1970) *J. Exp. Med.* **131**, 223–234.
12. Stutman, O., Yunis, E. J. & Good, R. A. (1968) *Proc. Soc. Exp. Biol. Med.* **127**, 1204–1207.
13. Denman, A. M. & Denman, E. J. (1970) *Clin. Exp. Immunol.* **6**, 457–472.
14. Zatz, M. M., Mellors, R. C. & Lance, E. M. (1971) *Clin. Exp. Immunol.* **8**, 491–500.
15. Dauphinee, M. J. & Talal, N. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 3769–3772.
16. Goldstein, A. L. & White, A. (1973) in *Contemporary Topics in Immunobiology*, eds. Davies, A. J. S. & Carter, R. L. (Plenum Publishing Corp., New York), pp. 339–350.
17. Goldstein, A. L., Slater, F. D. & White, A. (1966) *Proc. Nat. Acad. Sci. USA* **56**, 1010–1017.
18. Goldstein, A. L., Guha, A., Hardy, M. A. & White, A. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1800–1803.
19. Hooper, J. A., McDaniel, M. C., Thurman, G. B., Cohen, G. H., Schulof, R. S. & Goldstein, A. L. (1974) *Proc. N.Y. Acad. Sci.*, in press.
20. Bach, J. F., Dardenne, M. & Salomon, J. C. (1973) *Clin. Exp. Immunol.* **14**, 247–256.
21. Gershon, R. K. & Hencin, R. S. (1971) *J. Immunol.* **107**, 359–364.
22. Brunner, K. T., Mauel, J., Cerottini, J. C. & Chapuis, B. (1968) *Immunology* **14**, 181–196.
23. Proffitt, M. R., Hirsch, M. S. & Black, P. H. (1973) *Science* **182**, 821.
24. Mellors, R. C. & Huang, C. Y. (1966) *J. Exp. Med.* **124**, 1031.
25. Prosser, P. R. (1968) *Clin. Exp. Immunol.* **3**, 213.
26. Shirai, T. & Mellors, R. C. (1971) *Immunology* **12**, 179.
27. De Vries, M. J. & Hijmans, W. (1967) *Immunology* **12**, 179.
28. Goldstein, A. L., Guha, A., Howe, M. L. & White, A. (1971) *J. Immunol.* **106**, 773–780.
29. Scheid, M. P., Hoffman, M. K., Komuro, K., Hammerling, U., Boyse, E. A., Cohen, G. H., Hooper, J. A., Schulof, R. S. & Goldstein, A. L. (1974) *J. Exp. Med.* **137**, 1027–1032.
30. Bach, J. F., Dardenne, M., Goldstein, A. L., Guha, A. & White, A. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2734–2738.
31. Goldstein, A. L., Hooper, J. A., Schulof, R. S., Cohen, G. H., Thurman, G. B. & McDaniel, M. C. (1974) *Fed. Proc.*, in press.
32. Allison, A. C., Denman, A. M. & Barnes, R. D. (1971) *Lancet* **i**, 135.
33. Hardin, J. A., Chused, T. M. & Steinberg, A. D. (1973) *J. Immunol.* **111**, 650.