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Alteration in DNA Synthetic Response of Thymocytes from NZB Mice of Different Ages

(autoimmune disease/cytotoxicity)

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ABSTRACT **Thymocytes from NZB and DBA/2 mice were injected into lethally irradiated C57 Bl/6 recipients. DNA synthesis in spleen and lymph node was measured as the incorporation of radioactive 5-iodo-2-deoxyuridine. The generation of cytotoxic effector cells was also studied in an *in vitro* cytotoxicity assay, with EL-4 lymphoma target cells. The kinetics of DNA synthesis were similar for 2- and 8-week-old DBA/2 donors and for 2-week-old NZB donors with a peak on day 4. Thymocytes from 8-week-old and 9-month-old NZB donors showed a delayed onset of DNA synthesis which was still increasing on day 6. When NZB donor thymocytes from 2- and 8-week-old mice were mixed together before injection, as few as 25% of 8-week-old cells gave a DNA synthetic response characteristic of this aged population. These results suggest an abnormal thymocyte development in NZB mice which may relate to the subsequent emergence of autoimmune disease.**

New Zealand Black (NZB) mice are a model for spontaneous generalized autoimmune disease in humans (1, 2). NZB mice develop Coomb's positive hemolytic anemia, generalized lymphoproliferation, and immune complex nephritis and about 10% develop malignant lymphomas (1–3). Immunologic and viral factors, as well as genetic predisposition, play a role in the pathogenesis of their disease (4).

Antibody responses to many but not all antigens are increased in NZB mice (5–8). Immunologic unresponsiveness to bovine gamma globulin is difficult to achieve in 5 to 8-week-old NZB mice that do not yet manifest clinical disease (6). Unresponsiveness can be induced in 2 to 3-week-old mice but, unlike several control strains showing long-lived tolerance, NZB mice develop response by 2 months of age (9).

In normal mice, both bone marrow-derived (B cells) and thymus-derived (T cells) lymphocytes become unresponsive to foreign proteins (10). B-cell unresponsiveness requires larger amounts of antigen and is extremely short-lived, whereas T-cell unresponsiveness appears at lower antigen concentrations and is sustained (10). The rapid escape from tolerance in NZB mice could be explained if they failed to develop an unresponsive population of T cells.

Playfair found a raised threshold for tolerance induction to sheep erythrocytes in NZB mouse thymus cells (11). Jacobs *et al.* found that NZB thymocytes failed to develop and transfer cyclophosphamide-induced tolerance to sheep erythrocytes (12). Thus, T-cell unresponsiveness appears to be weak or absent in NZB mice.

Abbreviations: B and T cells, bone marrow-derived and thymus-derived lymphocytes, respectively.

Gershon has used an *in vivo* assay for thymocyte DNA synthesis to analyze response to histocompatibility and other antigens in normal mouse strains (13–15). Unresponsiveness can also be studied in this system (16). We have used his assay method to study DNA synthesis and generation of effector cells after injection of NZB thymocytes into lethally irradiated histoincompatible C57 Bl/6 recipients. We report an alteration in the kinetics of DNA synthesis occurring between 2 and 8 weeks of age which may relate to the subsequent immunologic abnormalities that develop in these mice.

MATERIALS AND METHODS

Mice. NZB mice were from our colony maintained at the Vivarium of the University of California, San Francisco. DBA/2 and C57 Bl/6 mice were from Jackson Laboratory, Bar Harbor, Maine.

Irradiation Procedure. C57 Bl/6 mice received 750 R total body x-irradiation from a Quadacondex Westinghouse x-ray machine with a 0.5-mm Cu and 1-mm Al filter at 15 mA and 230 kV. The dose rate was 122 R/min.

Thymus Cell Suspensions. Thymus cell suspensions were prepared by gentle mincing on sterile stainless steel mesh. The cells were washed twice in cold sterile RPMI 1640 medium (Microbiological Associates, Bethesda, Md.), which was used in all experiments reported here. Viable cells were counted by the trypan blue dye-exclusion method. 50×10^6 Viable thymus cells were injected intravenously in 0.5 ml into each recipient. All recipient mice were kept on acid water (17) for 1 week before experimentation. Four to eight recipient mice were used for each experimental determination. NZB and DBA/2 thymocyte donors were 2 weeks, 8 weeks, or 9 months of age and C57 Bl/6 recipients were 8 weeks of age. Three experiments were performed in which ratios of 8-week-old and 2-week-old NZB thymocytes (varying from 1–75% respectively) were mixed before injection into lethally irradiated C57 Bl/6 recipients. The total number injected was always 50×10^6 thymocytes for recipient animal.

DNA Synthesis. The procedure used was that of Gershon and Hencin (13) in which DNA synthesis is measured as uptake of ^{125}I-labeled 5-iodo-2-deoxyuridine (^{125}IdU, Amersham Searle Corp., Des Plaines, Ill., specific activity 4–6 μCi/g). Each mouse received 2 μCi of ^{125}IdU intraperitoneally in 0.2 ml of saline 24 hr before spleen and femoral lymph nodes were harvested. To determine the total isotope injected, 0.2 ml of

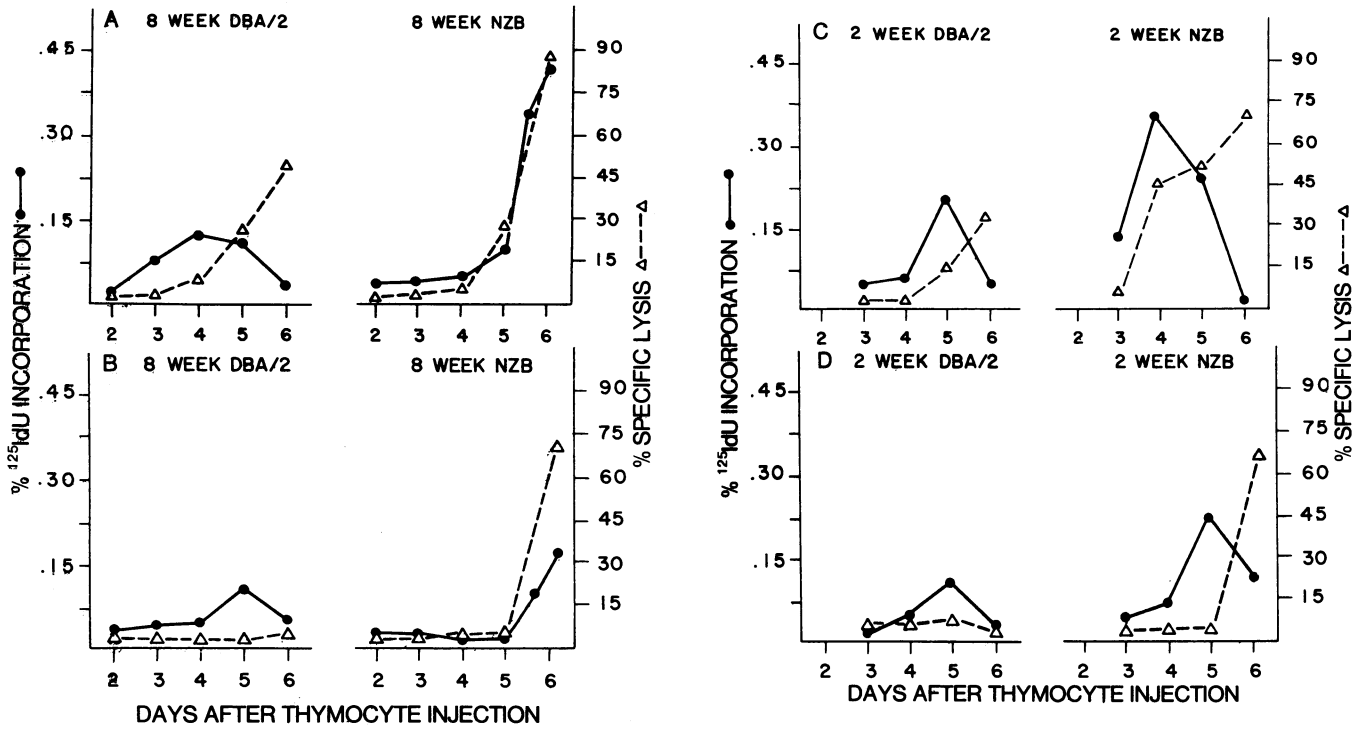

FIG. 1. DNA synthetic (% ^{125}IdU incorporation) and effector cell (% specific lysis) responses of spleen- and lymph node-seeking thymocytes from 2- and 8-week-old DBA/2 and NZB mouse donors. (*A* and *C*) spleen-seeking thymocytes; (*B* and *D*) lymph node-seeking thymocytes.

the ^{125}IdU solution was counted at the time of injection. Recipients receiving irradiated thymocytes (8000 R) did not incorporate any more isotope than recipients that had not been injected with thymocytes. Therefore, uninjected controls were used in the majority of experiments. A Packard model 5375 Gamma Scintillation Spectrometer was used for assessment of radioactivity. The % ^{125}IdU incorporated was determined according to the formula:

$$\frac{\text{organ (thymocyte-injected recipient)} - \text{organ (noninjected recipient)}}{\text{total isotope injected} - \text{organ (noninjected recipient)}} \times 100 = \%\,^{125}\text{IdU incorporation.}$$

Cytotoxicity Assay. The cytotoxic activity *in vitro* of sensitized lymphocytes was determined according to the method of Brunner (18) with slight modifications. Cytotoxicity was measured against EL-4 lymphoma target cells. To 2×10^7 EL-4 target cells, 200 μCi of [^{51}Cr]sodium chromate (ICN Chemical and Radioisotope Div., Irvine, Calif.) was added and the mixture was incubated for 30 min at 37° with occasional gentle shaking. The cells were then washed three times in 50-ml volumes of medium and adjusted to 10^6 cells per ml.

Sensitized lymphocytes were recovered from spleen and lymph nodes 2–6 days after injection of 50×10^6 donor thymocytes into lethally irradiated C57 Bl/6 recipients. The final lymphoid cell-to-target cell ratio was 100:1. The cell mixtures were placed in duplicate petri dishes, sealed in air-tight clear plastic boxes, and incubated at 37° for 8 hr in a mixture of 10% CO_2, 7% O_2, and 83% N_2. The boxes were rocked at a speed of 16 cycles/min. Specific cytotoxicity was determined by measuring the release of ^{51}Cr into the medium compared to maximal release (determined by water lysis) using the following formula:

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

RESULTS

Thymocytes from 8-week-old DBA/2 mice were selected as controls because they have the same H_2 type as NZB (H_2d) and because their DNA synthesis response is similar to other normal control strains (14). After injection of thymocytes into C57 Bl/6 irradiated recipients, the spleen- and lymph node-seeking populations were studied separately. In the spleen-seeking population, DNA synthesis as measured by ^{125}IdU incorporation began at day 3 and reached a peak at day 4 (Fig. 1*A*). Incorporation declined slightly at day 5 and was much less at day 6. Cytotoxicity appeared first at day 4 and increased in activity at days 5 and 6.

A very different DNA synthetic response was observed with 8-week-old NZB thymocytes (Fig. 1*A*). ^{125}IdU incorporation was markedly delayed and was first clearly evident at day 5, 24 hr after the peak in DBA/2. Incorporation increased greatly between days 5 and 6 and showed no tendency to decline during the observation period. The magnitude was greater than that found with DBA/2 thymocytes. The generation of cytotoxic lymphocytes was similar to DBA/2 but somewhat greater in magnitude. Similar but less striking results were obtained with lymph node-seeking thymocytes (Fig. 1*B*). DBA/2 synthesis peaked at day 5 and then declined, whereas NZB synthesis was only detectable at day 6. Cyto-

toxic activity was generated only by NZB thymocytes and not by DBA/2 cells.

Based on earlier experiments indicating major differences in tolerance induction between 2- and 8-week-old NZB mice (6, 9), we next studied younger animals. Thymocytes from 2-week-old DBA/2 mice responded like 8-week-old cells (Fig. 1C). ^{125}IdU incorporation peaked at day 5 and declined at day 6.

By contrast, 2-week-old NZB thymocytes were strikingly different from 8-week-old NZB thymocytes and now resembled more closely the DBA/2 response (Fig. 1C). ^{125}IdU incorporation was evident at day 3, peaked at day 4, declined at day 5, and was absent at day 6. The magnitude was again greater than the DBA/2. Cytotoxicity was present at day 4 and increased at days 5 and 6.

This difference between 2- and 8-week-old NZB thymocytes was also seen in the lymph node-seeking population (Fig. 1D). The NZB synthetic response was again similar to the DBA/2, peaking at day 5 and declining at day 6. Cytotoxicity was present only in the NZB at day 6.

Because age seemed to influence the kinetics of DNA synthesis by NZB thymocytes, we also studied older mice manifesting clinical disease (Fig. 2). The incorporation of ^{125}IdU by 9-month-old spleen-seeking thymocytes resembled that found with 8-week-old NZB cells. The lymph node-seeking cells now showed no incorporation. Cytotoxicity was not measured in this experiment but declines with age in NZB mice (19).

The difference in DNA synthetic response of 2- and 8-week-old NZB thymocytes made possible an experiment in which these two populations could be mixed in varying proportions and injected together into C57 Bl/6 recipient mice. We could thereby study the interaction of the two thymocyte populations and observe if one DNA synthetic response appeared to have a dominant influence. In the first experiment, three groups of recipient mice received 50×10^6 NZB thymocytes in which 8-week-old cells represented 75%, 50%, or 25% of the transferred population. In all three groups, the DNA synthetic response was characteristic of 8-week-old NZB thymocytes.

In a second experiment, 8-week-old NZB thymocytes comprised 25% or 10% of the tranferred population. In the former instance, the kinetics of DNA synthesis resembled that observed with 100% 8-week-old cells, whereas in the latter instance the kinetics resembled that observed with 100% 2-week-old cells (Fig. 3). In a third experiment, the 10% 8-week-old population gave an intermediate response, whereas 25% and 1% 8-week-old cells clearly resembled the 100% 8-week and 100% 2-week-old responses, respectively.

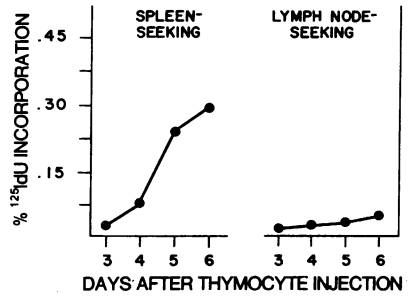

FIG. 2. DNA synthetic response of 9-month-old NZB thymocytes injected into lethally irradiated C57 Bl/6 recipients.

DISCUSSION

NZB mice demonstrate a progressive alteration and loss of T-cell effector functions throughout their life span. Young mice (less than 1 month of age) may have excessive effector activity, as judged by the ability to regress viral-induced neoplasms (20). Over the next several months, T-cell activity seems normal in comparison with other mouse strains. Thereafter, there is a progressive decline in effector activity, as measured by inability of spleen cells to induce graft-versus-host disease (21, 22), to respond *in vitro* to mitogens such as phytohemagglutinin (23, 24), and to regress sarcoma induced by the Moloney virus (20). There is also a loss of long-lived recirculating lymphocytes (25, 26).

The basis for these changes in T-cell activity is of great interest. NZB mice produce an auto-antibody that is cytotoxic for T cells, and has properties resembling a natural anti-theta antiserum (27). This natural thymocytotoxic antibody may play a role in altering T-cell effector function (28). There is a a slight decline in theta-positivity in older NZB mice (29–31), but probably not sufficient to account for these functional alterations. The present experiments suggest that the changes in peripheral T-cell function may arise secondary to an abnormality of thymocyte development already present at 8 weeks of age (before clinical disease). Such an abnormality might be genetically determined or induced by latent viral infection (4) and may relate to the tolerance abnormalities studied previously (6, 9). It is also present when clinical disease appears (e.g., at 9 months).

The unusual DNA synthetic response of 8-week-old NZB thymocytes was first called to our attention by Dr. Richard Gershon. Because of the different response to tolerogenic doses of bovine gammaglobulin shown by 2- and 8-week-old NZB mice, we decided to study these ages in detail. The dominant influence of 8-week-old thymocytes (when comprising only 25% of the donor population) was a surprise and is not easy to interpret. It recalls earlier experiments in which a T-cell subpopulation capable of amplifying graft-versus-host reactions was found deficient in older NZB spleens. This cell and full activity could be restored when as little as 1% of the grafted population was made up of young spleen cells (21).

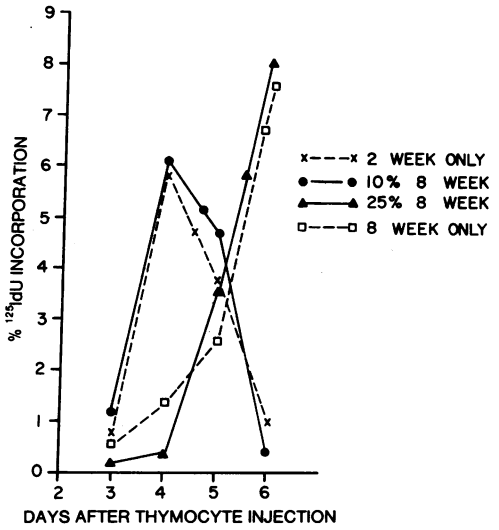

FIG. 3. DNA synthetic response of NZB thymocytes from 2- and 8-week-old donors mixed in various proportions before injection into lethally irradiated C57 Bl/6 recipients.

These studies, together with recent work on synergistic cooperation between T-cell subpopulations (32), suggest a possible interpretation for the kinetics of DNA synthesis by 8-week-old NZB thymocytes. Perhaps there is a premature exhaustion of a thymocyte subpopulation required for early initiation and termination of DNA synthesis. Future studies on thymocyte heterogeneity may lend support to this hypothesis. This phenomenon did not seem to influence the generation of effector cells, as judged by good cytotoxic activity.

It is suggested that the altered thymocyte DNA synthetic response may be a close reflection of a primary mechanism ultimately responsible for disease in NZB mice. If so, then attempts to correct the DNA synthetic response in various ways should be explored, for a suitable treatment of this type might also prevent or lessen the subsequent manifestations of autoimmunity.

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