Brief Definitive Report

NZB CYTOTOXIC LYMPHOCYTE RESPONSES

Kinetic Analyses

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Recently, we have been studying the regulation of cytotoxic lymphocyte (CTL) responses in NZB mice. These studies have demonstrated that NZB CTL were relatively resistant to suppressor signals (1). In the present paper, we studied the time-course of generation and the kinetics of lysis of CTL from nonprimed BALB/c, DBA/2, NZB, and alloantigen-primed BALB/c and NZB spleen cells in allogeneic mixed leukocyte cultures. These investigations suggested that NZB spleen cells have an accelerated generation of primary CTL responses, which, in some respects, mimic secondary CTL responses of alloantigen-primed mice. These observations shed light on recent reports of abnormal recognition phenomena by NZB T cells (2–4).

Materials and Methods

Mice. Female mice were used in all experiments. BALB/c (H-2d) and NZB/N (H-2o) mice were obtained from the breeding colonies of the National Institutes of Health, Bethesda, Md. DBA/2J (H-2d) and C57BL/6J (B6) (H-2b) mice were obtained from The Jackson Laboratory, Bar Harbor, Maine.

Alloantigen Sensitization In Vivo. Mice were sensitized by injection of \(1 \times 10^7\) B6 spleen cells, in 0.05 ml of Hanks' balanced salt solution (HBSS), into each hind footpad. After 14 d, sensitized mice were sacrificed by cervical dislocation, and their spleens were removed under aseptic conditions. Single spleen-cell suspensions were prepared by gentle teasing in HBSS. The spleen cells were either assayed for CTL activity or were used as primed responder cells in in vitro mixed leukocyte cultures (MLC).

MLC. Single spleen-cell suspensions were prepared in HBSS from in vivo primed mice and from nonprimed mice. B6 spleen cells, to be used as in vitro stimulator cells, were exposed to 1,500 rad of gamma irradiation. Cells were cultured in modified Eagle's minimum essential medium (MEM) with 10% heat-inactivated, mycoplasma-screened fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, N.Y.), in 16-mm, flat-bottom culture wells (Costar, Data Packaging, Cambridge, Mass.). 6–24 replicate wells that contained 1 \(\times 10^7\) responder cells and 1 \(\times 10^5\) irradiated stimulator cells were cultured in a final vol of 1.5 ml. Cultures were incubated for the specified number of days in a humidified atmosphere of 10% CO2, 7% O2, and 83% N2 at 37°C.

Preparation of Target Cells. EL-4 tumor cells were maintained by weekly intraperitoneal passage in B6 female mice. 2 \(\times 10^7\) tumor cells were incubated with 100 \(\mu\)Ci of \(^{51}\)Cr (Na\(^{51}\)CrO\(_4\), 200–500 Ci/g Cr sp act; New England Nuclear, Boston, Mass.) in 1 ml of HBSS with 10% FCS for 30 min at 37°C. The labeled target cells were then washed three times through 2 ml of FCS at 250 g for 10 min and diluted in MEM with 10% FCS to the desired concentration.

Cell-mediated Lympholysis Assay. Cytotoxicity was assayed as previously described (1). Effector cells were washed and resuspended in MEM with 10% FCS to the desired concentration of viable cells, and mixed with equal volumes (0.1 ml) of \(^{51}\)Cr-labeled target cells in serologic tubes. Effector and target cells were incubated for indicated times up to 4 h at 37°C in a humidified atmosphere with 5% CO2; radioactivity released into the supernate was measured.
in a gamma spectrometer (model 8000; Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Each determination was assayed in duplicate. Spontaneous \(^{51}\)Cr release was determined by incubating 0.1 ml of target cells with 0.1 ml of MEM with 10% FCS. Maximum release was determined by three cycles of freezing and thawing similar cultures. Spontaneous release was determined for each time-point in an assay. Data from 4-h fixed-end-point experiments are expressed as percent specific lysis and were calculated by the formula:

\[
\text{percent specific lysis} = 100 \times \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}}
\]

Data from multiple-end-point experiments are expressed as the number of target cells killed and were calculated by the formula:

\[
\text{No. of target cells killed} = \text{percent specific lysis} \times \text{number of target cells used in assay}.
\]

Analysis of Multiple-Time-Point Kinetic Data. The initial reaction velocities were determined directly from the slope of the plot of three data points within the 1st 90 min of the cytotoxicity assays. Each data point represented the mean of duplicate determinations; the standard error from the mean for each data point was <1%. The percent target cells killed in the initial 90 min of the cytotoxicity assays was determined from the slope of the plot of the initial velocity of lysis vs. the number of target cells used in the assay and were calculated by the formula:

\[
\text{percent target cells killed} = \frac{100 \times \text{number of target cells killed}}{\text{number of target cells used in assay}} + \text{time} = 100 \times \text{slope}.
\]

Linear regression correlation coefficients for the fit of the experimentally determined points to a straight line were between 0.96 and 0.99.

Results

Comparison of the Time-Course of Generation of BALB/c, DBA/2, and NZB CTL. BALB/c, DBA/2, and NZB spleen cells were stimulated in vitro with irradiated B6 spleen cells for 2, 4, or 6 d before assaying for anti-EL-4 CTL activity. BALB/c and DBA/2 CTL activity was first detected on day 4 and increased markedly by day 6. In contrast, initial NZB CTL activity was noted on day 2, markedly increased by day 4, and declined slightly by day 6 (Fig. 1). Similar CTL response curves were demonstrated with spleen cells from 1- and 6-mo-old NZB mice in three experiments. These primary NZB CTL response curves differed from those of primary BALB/c and DBA/2 CTL but were similar to the secondary CTL response curves of spleen cells from primed BALB/c mice (Fig. 1). Moreover, primary NZB CTL response curves were also similar to the secondary CTL response curves of spleen cells from primed NZB mice (Fig. 1).

Analyses of BALB/c, DBA/2, and NZB CTL Kinetics of Lysis after 4 and 6 d in MLC. To further compare the CTL responses, kinetic analyses were performed during the initial 90 min of the \(^{51}\)Cr-release assays. Nonprimed BALB/c, DBA/2, and NZB, and alloantigen-primed BALB/c and NZB, spleen cells were cultured with irradiated B6 spleen cells for 4 and 6 d. From these CTL responses, initial reaction velocities (\(V_i\)) were determined. A plot of the \(V_i\) (number of target lysed/h) against the corresponding initial target-cell number showed a linear dose-dependent relationship (Fig. 2). From the slope of these lines, the percent target cells lysed in the initial 90 min of assay (i.e., efficiencies of lysis) was determined. On day 4, primary NZB CTL had a \(V_i\) and efficiency of lysis only slightly greater than that of primary BALB/c or primary DBA/2 CTL, 15 ± 2% (mean ± SEM) vs. 8.6 or 7.6% killing (Table I). The \(V_i\) and efficiency of lysis of secondary BALB/c and secondary NZB CTL were much greater (Table I). On day 6, primary NZB, BALB/c, and DBA/2 CTL had similar \(V_i\) and efficiencies
Fro. 1. (Left) Time-course of generation of BALB/c, DBA/2, and NZB CTL in vitro. CTL activity of $10^6$ viable effector cells was measured before and every other day after in vitro stimulation with irradiated B6 spleen cells. Primary CTL response were generated from the spleen cells of BALB/c (○○), DBA/2 (■■), and 1-mo-old (▲▲) and 6-mo-old (▲▲) NZB mice. Secondary BALB/c (○○) and secondary NZB (▲▲) CTL responses were generated by the in vitro restimulation of spleen cells from mice B6-sensitized 14 d previously. Effector:target cell ratios were 20:1 in a 4-h $^{51}$Cr-release assay with EL-4 lymphoma target cells. Data are expressed as the mean percent specific lysis from triplicate determinations, as described in Materials and Methods. Standard errors were <2% of means.

Fro. 2. (Right) Plot of initial velocity of lysis by CTL from a 4-d MLC vs. target cell concentration. The velocity for BALB/c (△), NZB (○), and secondary BALB/c (○) CTL were determined from the number of target cells killed between 30 and 90 min in a $^{51}$Cr-release assay with labeled EL-4 lymphoma target cells and $10^6$ effector cells. Velocities at each target-cell concentration were obtained from data collected at three time points.

### Table I
Comparison of Kinetics of Lysis for CTL after 4 or 6 d in MLC

| Responder spleen cells* | Initial reaction velocity† | Percent cells killed in initial 90 min§ |
|------------------------|---------------------------|----------------------------------------|
|                        | Day 4 | Day 6 | Day 4 | Day 6 |             |
| BALB/c                 | 4.4   | 20.5  | 8.6   | 39    |             |
| DBA/2                  | 4.0   | 20.0  | 7.6   | 38    |             |
| NZB                    | 7.8   | 19.6  | 15    | 36    |             |
| Secondary BALB/c       | 28.6  | 32.5  | 55    | 62    |             |
| Secondary NZB          | 33.6  | 28.7  | 68    | 57    |             |

* $1 \times 10^6$ viable effector cells from a 4- or 6-d MLC were used in $^{51}$Cr-release assay with $5 \times 10^4$ labeled EL-4 lymphoma target cells.

† The number of target cells killed between 30 and 90 min. Data were calculated as described in Materials and Methods. Standard errors were <2%.

§ The efficiency of killing by the responder spleen cells. Data were calculated as described in Materials and Methods.
of lysis, killing 34–39% of the target cells (Table I). The secondary NZB and secondary BALB/c CTL had Vi and efficiencies of lysis that were similar to each other (Table I) and significantly greater than that of the primary NZB, BALB/c, or DBA/2 CTL (60 ± 2% vs. 37 ± 3%, P < 0.01). Because primary NZB, BALB/c, and DBA/2 CTL had values for Vi similar to each other, the greater killing observed with primary NZB CTL, compared with primary BALB/c or primary DBA/2 CTL during a 4-h 51Cr-release assay (Fig. 1), appeared not to result from different initial kinetics of lysis. Therefore, a further comparison on NZB and BALB/c CTL was done with multiple-time-point analyses throughout the entire 4-h assay. Again, their Vi were similar. However, the NZB CTL maintained their Vi twice as long as did the BALB/c CTL.

Discussion

Primary in vitro NZB CTL responses were initially detected on the 2nd d of MLC and peaked on the 4th d. In contrast, primary in vitro BALB/c and DBA/2 CTL responses were initially detected on the 4th d of culture and peaked on the 6th d. This accelerated time-course for the generation of primary in vitro NZB CTL was similar to that observed for secondary (i.e., in vivo primed, in vitro restimulated) NZB and BALB/c CTL. Furthermore, the spleen cells of 1- and 6-mo-old NZB mice gave similar primary in vitro CTL response curves.

These observations were extended by using multiple-time-point analyses during the early period of 51Cr-release assays to determine CTL kinetics of lysis. Comparison of the BALB/c, DBA/2, and NZB CTL kinetics of lysis demonstrated that (a) primary CTL responses could be easily distinguished from secondary CTL responses, and (b) the CTL generated from spleen cells of nonprimed NZB mice had hyperactive primary responses, not secondary responses.

The augmented primary immune response observed in the present study could have resulted from a defect in antigen nonspecific suppression, such as a defect in Ly-1+2+3+ feedback suppression (5, 6). Perhaps the relatively normal secondary NZB CTL responses observed herein, reflect normal antigen-specific regulatory mechanisms.

Recently, it has been reported that NZB spleen cells could generate a primary in vitro CTL response against unmodified non-NZB targets, which were identical to NZB mice in the major histocompatibility complex (i.e., H-2d) (2, 3). This phenomenon has been confirmed by others and found to be directed against Qr-1β-associated antigenic determinants (4) and, to a lesser extent, other minor locus determinants (U. Botzenhardt. Personal communication.). In normal strains, CTL directed against minor histocompatibility antigens are only observed in secondary immune response systems (7). We believe that the augmented primary CTL responses by NZB spleen cells in our studies could account for the apparent unusual recognition by NZB T cells. It may also explain the relative resistance of primary NZB CTL to suppressor signals (1), in contrast to the total resistance of secondary CTL to such suppressor signals (D. P. Huston and A. D. Steinberg. Manuscript in preparation.).

Thus, accelerated proliferation and differentiation, which characterize NZB B cells (8–10), also apply to NZB T cells. The resulting augmented primary CTL response is analogous to the augmented primary antibody response described by Katz et al. (11). Such an augmented primary T cell response may mimic secondary T cell responses.
with regard to the recognition of minor histocompatibility antigens. Our observations lead us to suggest that recognition by NZB cells of minor antigenic determinants does, in fact, not represent abnormal T cell recognition, but, rather, accelerated CTL kinetics.

Summary

Cytotoxic lymphocyte (CTL) responses of unprimed NZB spleen cells peaked on day 4 of culture as did cells from primed NZB or BALB/c mice. In contrast, primary BALB/c and DBA/2 responses peaked on day 6 of culture. Thus, NZB CTL generation was similar to the accelerated in vitro generation of CTL from the spleen cells of alloantigen-primed NZB and BALB/c mice.

To evaluate the kinetics of these CTL responses, multiple-time-point analyses were performed during the initial 90 min of the ^51^Cr-release assays. Analyses were done on days 4 and 6. On day 4, NZB CTL had an initial velocity of lysis slightly greater than that of BALB/c or DBA/2 CTL; however, it was far less than that of secondary NZB and secondary BALB/c CTL.

These studies indicate that NZB mice can generate primary CTL responses at an accelerated rate. Such augmented primary responses are unique and may explain recently described abnormal NZB T cell recognition as well as resistance of NZB CTL to suppressor signals.

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