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SPONTANEOUSLY ARISING CYTOTOXICITY TO THE P-815-Y MASTOCYTOMA IN NZB MICE

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SUMMARY

A spontaneously arising cytotoxic lymphoid cell in NZB mice is described. The cells, which appeared at about 9 months of age, were capable of killing the P-815-Y mastocytoma, but not a methylcholanthrene-induced sarcoma or chicken erythrocytes. Fractionation of NZB spleen cells indicated that the effector cell was neither phagocytic nor of T-cell origin. Antibody-dependent cytotoxicity in the NZB was then examined and found to be reduced when compared to the BALB/c. The role of this cell in the spontaneous cytotoxicity of the P-815-Y and its potential significance in the pathogenesis of autoimmune disease is discussed.

INTRODUCTION

Significant abnormalities in cell-mediated immunity have been described in ageing NZB mice. Their ability to induce graft-versus-host disease is impaired (Stutman, Yunis & Good, 1968; Cantor, Asofsky & Talal, 1970; Ghaffar, Krsiakova & Playfair, 1970), transformation of lymphocytes in response to PHA (Teague *et al.*, 1970; Leventhal & Talal, 1970) and the response to allogeneic cells in mixed lymphocyte culture (Leventhal & Talal, 1970) is reduced, and allograft rejection is delayed (Teague *et al.*, 1970). In the present study we describe another abnormal cell-mediated reaction in which lymphoid cells capable of killing the P-815-Y mastocytoma spontaneously appear in lymph node, spleen and peritoneal washings of older NZB mice. The significance of this fortuitous observation is discussed in relation to the NZB autoimmune syndrome.

MATERIAL AND METHODS

Mice

All NZB and BALB/c mice used for cytotoxicity studies were taken from an inbred colony described in previous publications (Ghaffar *et al.*, 1970). All other mice were obtained from the animal breeding unit of the Medical Research Council (Carshalton, Surrey).

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Tumour target cells

The P-815-Y ($H2^d$) DBA/2 mastocytoma was carried by intraperitoneal (i.p.) passage in DBA/2 or (DBA/2 × C3H) F_1 mice. A methylcholanthrene-induced sarcoma of BALB/c mice was also carried by i.p. passage.

Lymphoid cell suspensions

Mice were killed by cervical dislocation and their spleen and axillary, inguinal and mesenteric lymph nodes placed in Eagle's minimal essential medium (MEM) kept in an ice bath. The tissue was gently teased apart with forceps and the suspension allowed to settle for 45 min. The cell suspension was then washed three times and viable cell counts made in a haemocytometer chamber in the presence of Trypan Blue.

In some experiments phagocytic cells were removed from the spleen and lymph node. Iron powder (3 mg) was added to the lymphoid cells ($1{\cdot}5 \times 10^8$) and incubated 30 min at 37°C. Phagocytes which had ingested the iron particles were removed with a magnet (Greenberg *et al.*, 1973a).

Cytotoxicity testing

(a) *Spontaneous cytotoxicity.* Target cells were incubated with 100 μCi ^{51}Cr for 60 min at 37°C and washed four times in Eagle's MEM containing 5% foetal calf serum (FCS). After adjusting the cells to 5×10^4/ml, a sample of 200 μl was added to an equal volume of lymphoid cells ($2{\cdot}5 \times 10^6$/ml to 10×10^6/ml). The incubation tube was gassed with 5% CO_2 in a sealed desiccator and placed in a rocking incubator for 6 hr at 37°C. At the end of the incubation an additional 1 ml of Eagle's MEM was added, the contents of the tube mixed thoroughly, and spun at 2000 rpm for 5 min. A sample of 800 μl was removed and both aliquots counted. Cytotoxicity was assessed by the following formula:

$$\frac{\%\,^{51}Cr \text{ released in the sample} - \%\,^{51}Cr \text{ released in the control}}{\%\,^{51}Cr \text{ released in distilled water} - {}^{51}Cr \text{ released in the control}} \times 100.$$

Controls were calculated from the spontaneous release of ^{51}Cr in an incubation of 5×10^4/ml labelled target cells and unlabelled target cells in a comparable concentration to the lymphoid cells used in the experiment.

(b) *Cytotoxicity of cells from immunized mice.* Tumour cells (3×10^7) were injected i.p. into NZB or BALB/c mice. Eleven days later the mice were killed and their spleens removed. Lymphoid cells were prepared and their cytotoxicity tested as outlined above.

(c) *Cytotoxicity of cells sensitized by allogeneic transfer.* Three-month-old NZB or BALB/c mice were irradiated (800 rad) and given 5×10^7 washed spleen cells or 2×10^7 washed lymph node cells intravenously (i.v.). Spleens were removed at day five after transfer and tested as outlined above.

(d) *Cytotoxicity of normal lymphoid cells to antibody-coated chicken erythrocytes (CRBC).* Details of this assay have been given previously (Perlmann & Perlmann, 1970; Greenberg, Shen & Roitt, 1973a). Rabbit anti-CRBC antibody (1/30,000), normal lymphoid cells freed of phagocytes with iron powder, and ^{51}Cr-labelled CRBC are incubated for 18 hr at 37°C in Eagle's MEM containing 5% FCS. Supernatant and cells are separated after centrifugation and the aliquots counted. Cytotoxicity was calculated by the formula outlined above.

RESULTS

Spontaneous cytotoxicity of NZB lymphoid cells for P-815-Y mastocytoma

Fig. 1 illustrates the spontaneous P-815-Y cytotoxicity detected in spleen and lymph node cells in NZB mice of various ages. Cytotoxicity was most pronounced both in incidence and level at 9 months of age. Some activity was seen at 6 months and 12 months, but little or no cytotoxicity was detected in the very young or very old NZB mice. The appearance of cytotoxic cells was quite sporadic. The incidence of positives (>10% cytotoxicity) was

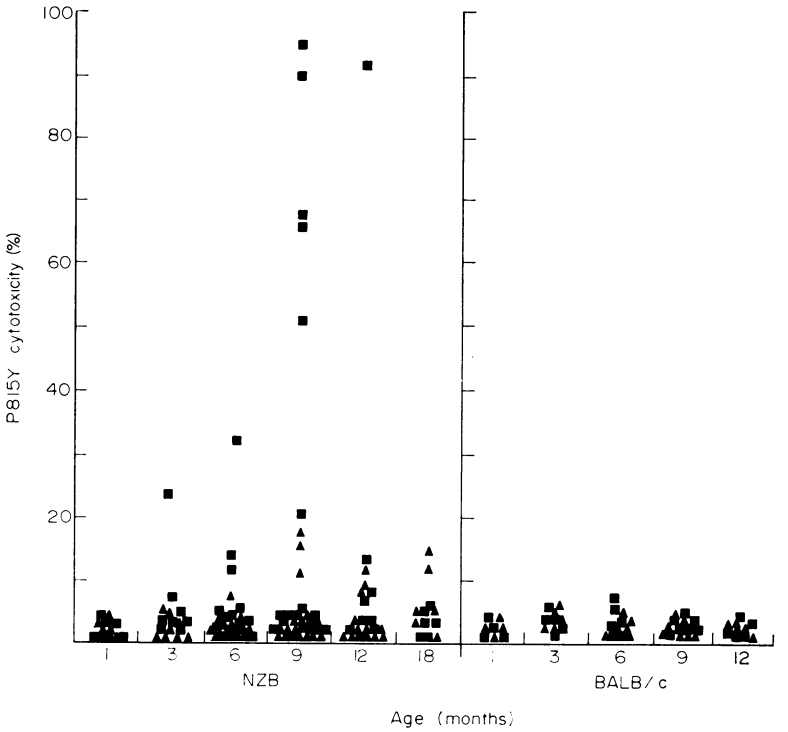

FIG. 1. Spontaneous cytotoxicity for the P-814-Y mastocytoma detected (▲) in spleen and (■) lymph node of NZB mice of various ages compared to the cytotoxicity of BALB/c spleen and lymph node. Observations represent either individuals or pools of two or three mice tested at a lymphoid to target cell ratio of 200:1.

about 15 to 20% in the 9-month-old group and much less at other ages. This figure may, in fact, be considerably lower, since many samples were tested in pools of two or three mice each. No cytotoxicity was detected in BALB/c mice of any age (Fig. 1). C3H and C57Bl spleen and lymph node cells were also examined in 9-month-old and 1-month-old mice, and again no activity was found.

Lymph node cells exhibited a greater incidence of positives as well as a higher level of cytotoxicity than spleen cells. Spleen cells in mice with positive lymph nodes were usually negative and little activity could be detected in the lymph nodes of mice with positive spleen cells (Fig. 2). In several experiments, peritoneal washings exhibited cytotoxicity comparable to that found in lymph node.

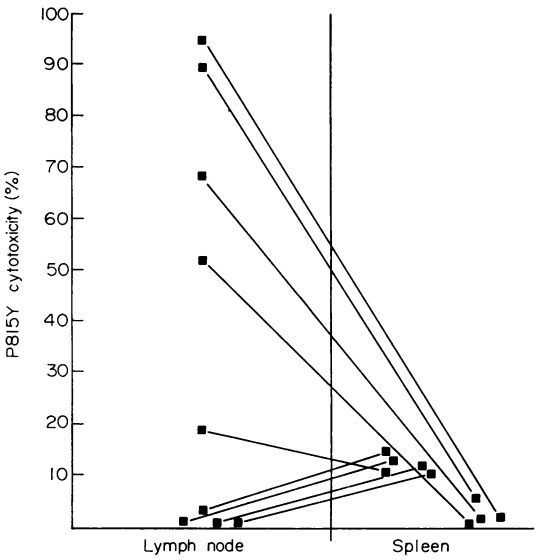

FIG. 2. Comparison of spontaneous cytotoxicity for the P-815-Y in spleen and lymph node of the same NZB mouse. All observations are made at a lymphoid to target cell ratio of 200:1.

The cytotoxicity was detected most readily at lymphoid to target cell ratios of 200:1 or greater, however, activity rapidly decreased and was not detected at ratios $<100:1$, even in very active lymph node preparations (Fig. 3). In contrast, the T cell cytotoxic for the P-815-Y found in C3H spleen 12 days after i.p. immunization (Miller *et al.*, 1971), is still detectable at ratios of $<6:1$.

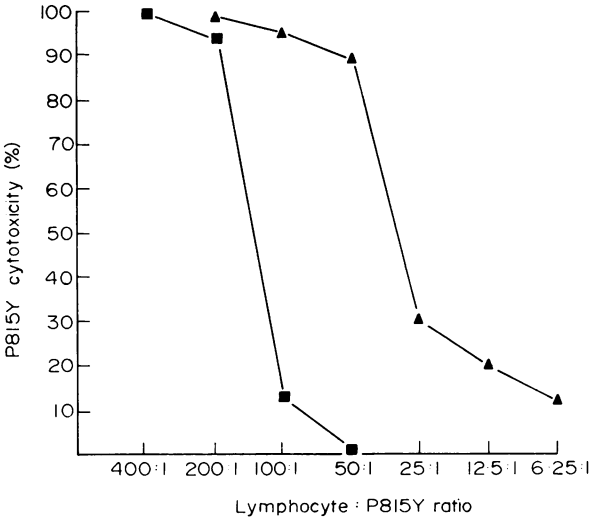

FIG. 3. Comparison of the spontaneous cytotoxicity of (■) NZB lymph node cells, to (▲) immune C3H spleen taken 12 days after i.p. immunization with 3×10^7 P-815-Y, at various lymphoid to target cell ratios.

Recalling the experiments of Playfair & Krsiakova (1969) in which spleen cells from older NZB mice were capable of inducing a GVH-like response on transfer into syngeneic neonatal mice, an attempt was made to expand the cytotoxic cell population by a similar transfer. Fig. 4 illustrates the results of transferring either spleen or lymph node cells from 1-month-old or 6-month-old NZB mice into lethally irradiated (800 rad) 3-month-old syngeneic recipients. The incidence of positives in the transferred cells is slightly greater than that seen in the untreated mice of a comparable age. A similar transfer of 6-month-old BALB/c spleen and lymph node into 3-month-old irradiated syngeneic mice did not produce any cytotoxic cells. BALB/c mice are, however, capable of being immunized with the P-815-Y so that their cells are cytotoxic *in vitro*.

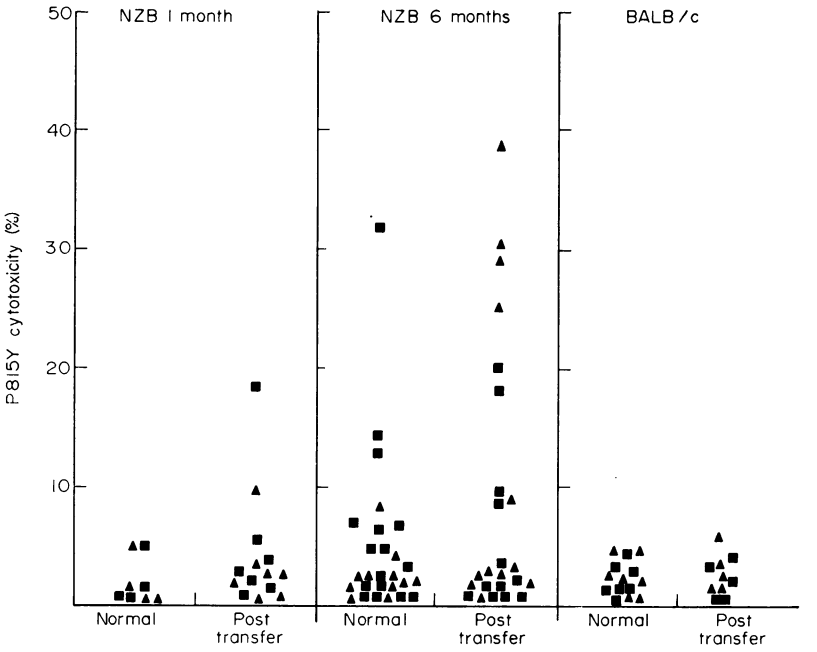

FIG. 4. Cytotoxicity of spleen cells after transfer of (▲) syngeneic 6-month or 1-month spleen or (■) lymph node into lethally irradiated (800 rad) 3-month-old NZB or BALB/c mice. This is compared to the spontaneous cytotoxicity found in a group of normal NZB and BALB/c mice of comparable age to the donors.

Specificity of the P-815-Y cytotoxicity

Because of the possibility that the observed cytotoxicity for the P-815-Y was the result of a non-specific response to an unrelated antigen, 3-month-old NZB and BALB/c mice were challenged with a variety of soluble and cellular antigens in an attempt to induce cytotoxicity non-specifically against the tumour. Keyhole limpet haemocyanin (100 μg) in Freund's complete adjuvant was injected subcutaneouly, and spleen and lymph node obtained 14 days later. Oxazolone-stimulated (Ptak & Asherson, 1969) lymph node and spleen cells were taken 10 days after skin application. Cells were also obtained from mice 12 days after receiving 3×10^7 C3H (H_2^k) or C57Bl (H_2^b) spleen lymphoid cells i.p. Neither the NZB nor

BALB/c mice immunized against these antigens exhibited cytotoxicity to the P-815-Y (H_2^d) mastocytoma.

Nine-month-old NZB and BALB/c lymph node cells were then tested for cytotoxicity against two other target cells, a methylcholanthrene-induced sarcoma of BALB/c (H_2^d) mice and normal chicken erythrocytes. No activity was detected against either target cell.

Immunization with the P-815-Y mastocytoma

The observation that the NZB mice may be hyperreactive to cellular antigens (Playfair, 1968; Evans, Williamson & Irvine, 1968) raised the possibility that an otherwise insignificant cross-reaction between an environmental antigen and the P-815-Y may have been amplified to detectable levels. If this were the case one might expect the response of the NZB mice to direct challenge with P-815-Y to be greater than the BALB/c mice. This, however, was not the case as both the BALB/c and NZB mice responded in a very similar manner to immunization with the tumour (Fig. 5).

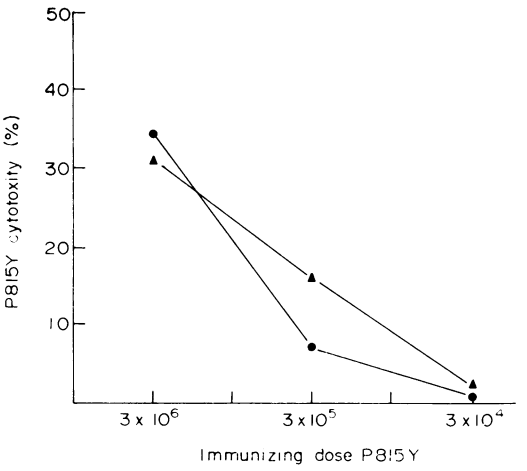

FIG. 5. Cytotoxicity of (▲) 3-month-old NZB (H_2^d) and (●) BALB/c (H_2^d) spleen cells 12 days after intraperitoneal immunization with decreasing doses of P-815-Y (H_2^d) mastocytoma. Cells were tested at a lymphoid to target cell ratio of 100 : 1. Each point represents the mean cytotoxicity of four mice.

Characterization of the spontaneous cytotoxic cell

Lymph node cells from 9-month-old NZB mice were examined for cytotoxicity before and after removal of phagocytic cells with iron powder (Table 1). No significant effect on the cytotoxicity was detected. Lymph node cells were then treated with heterologous anti-T antiserum and complement (Gyöngössy & Playfair, 1973) or normal rabbit serum and complement. In two separate experiments no effect was noted on cytotoxicity even though the antiserum completely abolished the activity of C3H spleen cells immunized with the P-815-Y.

The antibody-dependent cytotoxic cell of the NZB mice

With the observation that the spontaneous P-815-Y cytotoxicity was not due to either a

TABLE 1. The effect of heterologous anti-T antiserum and complement and the removal of phagocytes on NZB cytotoxicity for the P-815-Y mastocytoma

Treatment	Cytotoxicity * (%)
None	29·3
Iron powder	27·6
Anti-T and complement	27·5
Normal rabbit serum and complement	30·3
None	20·1
Iron powder	20·5
Anti-T and complement	14·4
Normal rabbit serum and complement	15·1
None	67·7
Iron powder	66·7

* Lymph node cells from 9-month-old NZB mice were tested at 200:1 lymphoid to target cell ratio.

T cell or a phagocytic cell, the antibody-dependent cytotoxic cell (Perlmann & Perlmann, 1970; Greenberg *et al.*, 1973) of the adult NZB mouse was examined. Spleen cells were obtained from NZB and BALB/c mice and freed of phagocytes. The cytotoxicity of these

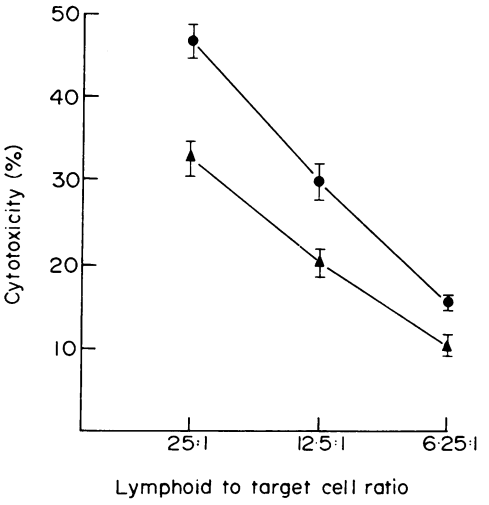

FIG. 6. Cytotoxicity (±SEM) of (▲) normal adult NZB and BALB/c (●) spleen cells for antibody-coated chicken erythrocytes. Significant differences ($P < 0{\cdot}005$) were found between the two strains at all lymphoid to target cell ratios.

cells was tested against chicken erythrocytes coated with rabbit anti-chicken erythrocyte antiserum. The ability of NZB lymphoid cells to kill antibody-coated erythrocytes was found to be significantly lower ($P<0{\cdot}005$) than the BALB/c at all lymphoid to target cell ratios examined (Fig. 6).

DISCUSSION

The cytotoxic cell described in this paper was discovered fortuitously while investigating the ability of older NZB mice to induce a GVH disease on transfer into syngeneic neonatal mice (Playfair & Krsiakova, 1969). The cytotoxicity proved quite difficult to characterize because of its sporadic nature. Several experiments and many mice were often required to make a single positive observation. The cytotoxic cell was, however, partially characterized and some information was obtained about its specificity.

The phenomenon appears to be similar in many respects to other types of cytotoxicity. The cytotoxic cells exhibited a typical dose–response dilution curve when compared to either the cytotoxic T cell or the antibody-dependent cytotoxic cell. These cells also were detectable in sites similar to both other cytotoxic cells; lymph node, spleen and peritoneal cavity. They were, however, present in much lower concentration since very high lymphoid to target cell ratios were required to detect them adequately.

The cytotoxicity of the cells on transfer into syngeneic recipients was slightly increased; however, this was minimal and the interpretation unclear. Although it is tempting to speculate about the role of these cells in the GVH-like disease reported by Playfair & Krsiakova (1969), there is no evidence that the cell showing spontaneous cytotoxicity for the P-815-Y was involved in this type of reaction.

The effector cell appears to be similar to the antibody-dependent cytotoxic cells being studied by many investigators (Perlmann & Perlmann, 1970; McLennan & Harding, 1970a; Greenberg *et al.*, 1973a and b). Recent investigations have indicated that this cell is neither a T nor B lymphocyte but most probably of the monocyte series (Greenberg *et al.*, 1973b). It is not clear at this time whether other effector cells exist, perhaps belonging to a new class of lymphoid cells. It does seem certain, however, that this type of cytotoxicity requires antibody, either on the surface of the target cell (Perlmann & Perlmann, 1970) or bound to the surface of the effector cell in the form of a cytophilic antibody (Perlmann & Perlmann, 1970), or a complex (Perlmann, Perlmann & Biberfield, 1972; Greenberg, Shen & Roitt, 1973c).

In the present study, the NZB antibody-dependent cytotoxic cell, examined by its ability to kill antibody-coated CRBC, proved to be less efficient than the BALB/c. Recently, DeJesus, Holborow & Brown (1972) reported a significant decrease in the ability of NZB cells to transport aggregated immunoglobulin to germinal centres, and attributed this finding to a deficiency of non thymus-derived lymphoid cells. Since cells other than B lymphocytes have Fc receptors, it is possible that the cytotoxic cell is also an important carrier of complexes. The defect seen in the recognition of complexes for transport to germinal centres may be comparable to the decreased ability of these cells to recognize an antibody–target cell complex. It is not clear, however, whether this is due to a reduction in the number of effector cells, or a decrease in the availability of the Fc receptor. The latter hypothesis is appealing both because it is well known that irrelevant complexes easily bind to the Fc receptor and block cytotoxicity (McLennan, 1972), and that the NZB has large amounts of

renal and, probably, circulating complexes (Mellors *et al.*, 1971; Tonietti, Oldstone & Dixon, 1970; Linder & Edgington, 1973). If these complexes are formed with antibody capable of recognizing the P-815-Y, the cytotoxic cells would be specifically armed (Perlmann *et al.*, 1972; Greenberg *et al.*, 1973c) to recognize the target cell.

The effector cell, or more probably the antibody through which it acts, is apparently able to recognize an antigen specific for the P-815-Y as it was not possible to reproduce the cytotoxicity by immunization with other antigens, or demonstrate cytotoxicity for other target cells. Since the mice had not been exposed to the tumour, one must assume that sensitization occurred either with a cross-reacting environmental antigen or with a shared autoantigen to which the mouse had lost tolerance.

Immunization with viral agents produces humoral and cell-mediated protection against tumours bearing those viral antigens (Lindemann & Klein, 1967; Axler & Gircurdi, 1970; Beverley, Lowenthal & Tyrrell, 1973). Since the P-815-Y target cell was passaged in mice living in the same colony it is possible that it carried the same virus to which the NZB had become sensitized. Why this should occur only in the NZB and not the BALB/c mice, and why it was limited to mice of one age group, is difficult to explain. The NZB did not differ from the BABL/c mice in response to immunization with the P-815-Y antigen so the NZB cytotoxicity could not be accounted for by hyperreactivity to the viral antigen. Since it is well known that the NZB develops a wide variety of autoantibodies (Bielschowsky, Helyer & Howei, 1959; Aarons, 1964; Norins & Holmes, 1964), and that these tend to appear later in its life (Ghaffer & Playfair, 1971), the second possibility that the NZB and P-815-Y share an autoantigen must be considered. The antigens of the Gross virus are particularly attractive since the NZB loses tolerance to these antigens late in life and produces detectable circulating antibody (Mellors, Aoki & Huebner, 1969). This antibody could then provide the vehicle through which the cytotoxic cell acts. Either it is produced *in vitro* during the cytotoxicity assay culture and attaches to the target cell (MacLennan & Harding, 1970b), or it binds to the effector cell *in vivo* as an antigen–antibody complex which then recognizes the target cell through its free antibody valences (Greenberg *et al.*, 1973c). Other autoantigens could also be involved, such as the Murine Leukaemia Virus antigen (Nowinski *et al.*, 1968; Mellors *et al.*, 1969). Although these possibilities are speculative, the role of the antibody-dependent cytotoxic cell armed with complexes containing autoantibodies against specific cell surface antigens may be of considerable importance in the pathogenesis of autoimmune disease.

In summary, cytotoxic cells of non-T cell origin were found in older NZB mice which were capable of recognizing a specific antigen on the P-815-Y mastocytoma. It is suggested that the most likely explanation for this unusual phenomenon is the loss of tolerance to an antigen common to both the NZB and the P-815-Y.

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