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# THE *IN VITRO* ACTIVITY OF PERITONEAL EXUDATE CELLS FROM NEW ZEALAND BLACK MICE (NZB/B1) IN THE PRESENCE OF $I^{125}$-BSA (BOVINE SERUM ALBUMIN)

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SUMMARY

Peritoneal exudate cells from old NZB/B1 mice were found to be more active in taking up $I^{125}$-labelled bovine serum albumin than similar cells from age matched BALB/c, C57B1 and CBA mice. The cells from both young and old NZB/B1 mice were markedly less able to degrade the ingested antigen than cells from the other strains. These observations are discussed in relation to the development of autoimmune phenomena in the NZB/B1 strain.

## INTRODUCTION

The New Zealand Black (NZB/B1) strain of mice has provided immunologists with a convenient experimental model for the study of the immunopathology of auto-immune disease. The numerous studies on these mice fall into two main categories:

(1) Those suggesting a disturbance of the immunological mechanism with an underlying genetic defect and possibly associated with deficiency of thymic activity (Bielschowsky & Bielschowsky, 1964; Holmes, Gorrie & Burnet, 1961; Holmes & Burnet, 1964; Holmes & Burnet, 1966a; Holmes & Burnet, 1966b).

(2) Where the disturbed immunological mechanisms are chiefly brought about by an extrinsic agent such as a virus (Barnes & Tuffrey 1969; East, Prosser, Holborow & Jaquet, 1967; East & Branca, 1969; Lambert & Dixon, 1970; Mellors & Huang, 1966; Mellors & Huang 1967; Mellors, 1969).

The nature of the abnormal activity of the immunological mechanism has yet to be elucidated. These mice are known to be hyper-responsive to protein antigens (Staples & Talal, 1969; Playfair, 1968; Weir, McBride & Naysmith, 1968) and to have depressed cellular immunity (Stutman, Yunis & Good, 1968) and to respond poorly to mitogens such

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as PHA (Leventhal & Talal, 1970) which is said to be a thymus-dependent activity (Adler *et al.*, 1970).

Apart from the low *in vitro* response to PHA (Leventhal & Talal 1970), little is known about the activity of the individual cell components of their immune system and the present study is an attempt to determine how NZB/Bl peritoneal exudate (PE) cells behave with respect to their ability to take up and degrade antigen compared with other strains of mice. A further report will present the results of an investigation of the activity of NZB/Bl spleen cells and lymph node cells.

## MATERIALS AND METHODS

### *Mouse strains*

NZB/Bl and BALB/c were the main strains used. C57/Bl and CBA mice were also used. The NZB/Bl mice were obtained from Carshalton or provided by Dr E. J. Holborow (Taplow) and bred on the traffic light system. The BALB/c mice were obtained from Dr June East (Clinical Research Centre, Stanmore). The CBA and C57Bl mice were from stock kept in the Animal House of the Bacteriology Department.

The mice were grouped, according to age, into two groups: (1) those aged 2–4 months; (2) those aged 13–16 months.

Animals of both sexes were included in both groups.

### *Tissue culture*

Peritoneal exudate (PE) cells were harvested from the mice after intraperitoneal injection of 2 ml Eagles minimal essential medium (MEM) containing heparin (100 U/ml) and their concentration adjusted to $1 \times 10^6$ cells/ml. The cells were distributed among Mackaness culture chambers which had been sterilized by UV irradiation. All procedures were carried out under sterile conditions and using a sterile cabinet for changing the medium. The cells were incubated in the Mackaness chambers at 37°C in air for 1 hr to allow the macrophages to adhere to the glass coverslips. At the end of this period, the cell sheets were washed, *in situ*, three times with Eagles MEM.

### A. *Uptake of $I^{125}$-BSA*

Eagles MEM containing 20% dialysed lactalbumin (Difco T.C. Lactalbumin hydrolysate) and BSA (labelled with $I^{125}$ by the method of Hunter & Greenwood 1962) at a concentration of approximately 100 $\mu$gN/ml with a specific activity of 0·05–0·5 $\mu$Ci/$\mu$g N. (BSA was obtained from Armour Chemicals Ltd) and was added to completely fill the chambers (0·85–0·90 ml per chamber). The chambers were then incubated at 37°C in 5% $CO_2$ in air for periods ranging from 2–24 hr. Control cultures were set up in which:

(a) the cells were incubated at 4°C for up to 24 hr to determine the amount of $I^{125}$-BSA taken up passively by the cells. These controls were reincubated at 37°C for 1 hr before the uptake of $I^{125}$-BSA was determined in order to permit the macrophages to re-adhere to the glass; and

(b) there were no cells—to determine the amount of isotope which adhered to the glass.

After the required length of time in the $I^{125}$-BSA Eagles medium, the cell sheets were washed, *in situ*, five times with Eagles MEM, the coverslips bearing the cell sheets fractured and the pieces of glass placed into Wasserman tubes. The uptake of $I^{125}$-BSA was deter-

mined by estimating the $\gamma$-emissions in a Nuclear Enterprises Gammamatic Mark I Gamma counter.

B. *Degradation of $I^{125}$-BSA*

Cultures were set up as in A and incubated at 37°C in 5% $CO_2$ in air for 16 hr. The cell sheets were then washed, *in situ*, five times with Eagles MEM. Several chambers were removed from the experiment at this time, for use as controls, to record the uptake of the $I^{125}$-BSA by the cells during the 16-hr incubation period. The rest of the chambers were filled with Eagles MEM containing 20% lactalbumin and 0·01 M tyrosine (BDH-L-tyrosine) for periods of up to 24 hr, in 5% $CO_2$ in air at 37°C. Controls, however, were incubated at 4°C to determine the amount of $I^{125}$-BSA lost passively over this period. At the end of the incubation period in this $I^{125}$-BSA—free medium, the supernate was removed from the culture chambers and treated with 20% trichloracetic acid (TCA) w/v, for 1 hr at 4°C. The TCA-treated supernates were centrifuged at 2500 rev/min at 4°C in an MSE Major centrifuge for 20 min and the non-precipitable portion separated into fresh Wasserman tubes, leaving the precipitated fraction (including the undegraded BSA) behind in the first tubes. (Ehrenreich & Cohn 1967).

The percentage of $I^{125}$-BSA which had been degraded (i.e. that BSA which was not precipitated by TCA) by the macrophage was determined, again using the Mark I Gammamatic. The coverslips bearing the cell sheets were fractured and the amount of radioactivity retained by the cells estimated in the Gammamatic.

*Uptake of $I^{125}$-BSA in the presence of parafluorophenylalanine* (Cohn & Benson, 1965; Cohn, 1966)

Parafluorophenylalanine (250 $\mu$g/ml) was incorporated into the Eagles MEM medium supplemented with 20% lactalbumin and containing $I^{125}$-BSA. The uptake experiment was carried out as in A to determine whether or not the uptake of $I^{125}$-BSA by the macrophage was an active process.

*Coombs' test*

The direct Coombs' test (DCT) was performed on erythrocytes collected from the retro-orbital plexus and washed five times with phosphate-buffered saline. A rabbit anti-mouse globulin, prepared in NZW rabbits, absorbed of all its anti-erythrocyte activity, was used to detect globulin on the erythrocytes.

## RESULTS

*The uptake of $I^{125}$-BSA cells in the presence of parafluorophenylalanine*

As can be seen from Fig. 1, significantly less isotope-labelled BSA was taken up by the macrophages when cultured in the presence of parafluorophenylalanine than in its absence, thus supporting the view that the isotope-labelled antigen was taken up by an active process (Cohn & Benson, 1965; Cohn, 1966).

*The uptake of $I^{125}$-BSA by PE cells*

(a) *Comparison between NZB/B1 mice aged* 2–4 *months and NZB/B1 mice aged* 13–16 *months*

Throughout the whole 24 hr of incubation, there was a highly significant difference ($P<0{\cdot}001$) between the two curves (see Fig. 1). An interesting feature, at present inexplicable, was the peak of activity observed in both groups of mice between 4 and 6 hr incubation. This result was a consistent finding in every experiment using the two groups. Overall, the PE cells of the older mice showed a much higher activity.

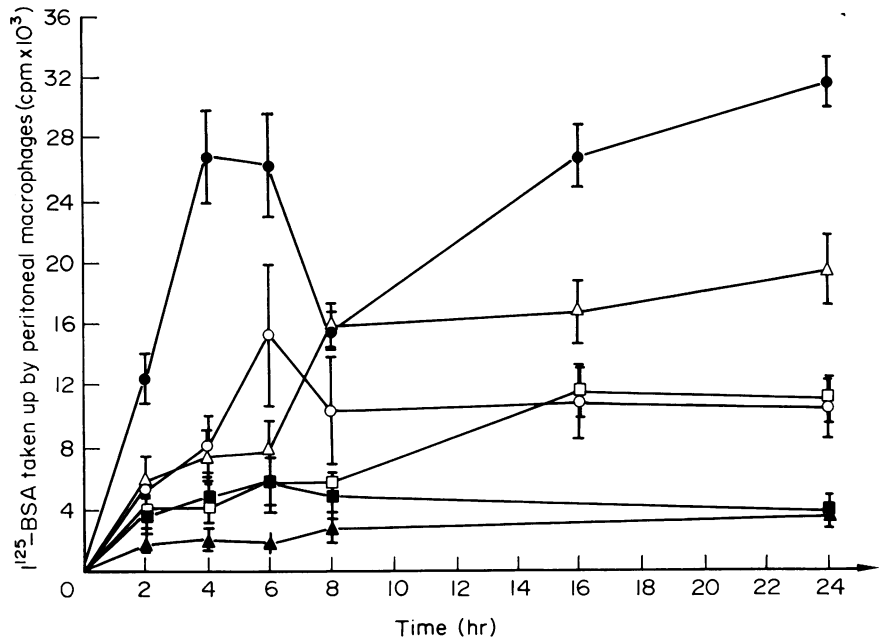


FIG. 1. The uptake of $I^{125}$-BSA by peritoneal exudate cells at 37°C in 5% $CO_2$ in air over 24 hr. Standard deviations included. Source of peritoneal exudate cells: (○) C−ve NZB/B1 mice aged 2–4 months. (●) C+ve NZB/B1 mice aged 13–16 months. (■) C+ve NZB/B1 mice—parafluorophenylalanine incorporated into the medium. (□) BALB/c mice aged 2–4 months. (△) BALB/c mice aged 13–16 months. (▲) BALB/c mice aged 13–16 months—parafluorophenylalanine incorporated into the medium.

(b) *Comparison between BALB/c mice aged* 2–4 *months and BALB/c mice aged* 13–16 *months*

Here again the PE cells from the older mice showed a higher activity than those of the younger mice, with highly significant differences from 8 hr incubation onwards ($P<0{\cdot}001$).

(c) *Comparison between CBA mice aged* 2–4 *months and CBA mice aged* 10–12 *months and between C57 Bl mice aged* 2–4 *months and C57 Bl mice aged* 8–12 *months*

The same pattern emerged; the PE cells from the older mice showed a higher activity than did those of the younger animals at 24 hr incubation. The CBA mice gave figures for the uptake of labelled-BSA of $3{\cdot}2\times10^3$ and $8{\cdot}5\times10^3$ cpm for mice aged 2–4 months and 10–12 months respectively; the C57/B1 gave figures of $10{\cdot}4\times10^3$ and $15{\cdot}9\times10^3$ cpm at 2–4 months and 8–12 months of age respectively. The differences were significant in both cases ($P<0{\cdot}01$).

(d) *Comparison between NZB/B1 mice aged 2–4 months and BALB/c mice aged 2–4 months*

Up until 8 hr incubation with the labelled-BSA, there was a significantly higher uptake of $I^{125}$-BSA by the PE cells of the NZB/B1 mice, whereas at 16 and 24 hr the differences were no longer significant ($P > 0{\cdot}05$).

(e) *Comparison between NZB/B1 mice aged 13–16 months and BALB/c mice aged 13–16 months*

Except at 8 hr, where the difference between the activity of PE cells of these two groups was not significant, there was a highly significant difference in the uptake of $I^{125}$-BSA by these cells throughout the 24-hr incubation period, with the higher activity in the NZB/B1 group. The NZB/B1 mice in this age group were all Coombs' positive (C+ve).

(f) *Comparison between NZB/B1, BALB/c, CBA and C57B1 mice aged 2–4 months at 24 hr of incubation in labelled BSA*

The CBA mice gave the lowest results, significantly lower than the C57 B1 mice which had the second lowest activity. However, the C57 B1 results were not significantly different from those of the NZB/B1 and BALB/c strains at that point of time.

(g) *Comparison between NZB/B1, BALB/c CBA and C57 B1 mice of the older age groups at 24 hr incubation in labelled BSA*

Once again the CBA mouse PE cells took up least $I^{125}$-BSA over the 24-hr period; this result was followed by that of the C57 B1 mouse PE cells which, this time, yielded a result significantly lower than the next group—the BALB/c mice. The maximum activity was observed in the NZB/B1 group whose PE cells took up approximately 1·6 times as much $^{125}$BSA as did the BALB/c PE cells. The CBA and C57 B1 groups continued to show the difference in activity between the different age groups within the strains; cells of the older mice giving a higher activity than those of younger ones.

B. *Loss and degradation of $I^{125}$-BSA by peritoneal exudate cells over 24 hr in $I^{125}$-BSA—free medium following 16-hr pulse labelling with $I^{125}$-BSA*

(a) *The percentage of $I^{125}$-BSA initially incorporated into the PE cells during pulse labelling which was lost by the cells to the medium when cultured in $I^{125}$-BSA-free medium*

Fig. 2 shows that both the young and old BALB/c mouse cells lost significantly more of the labelled BSA than did the corresponding NZB/B1 groups. Cells from the 2–4-month-old CBA mice lost 71·43% of the isotope and those from 10–12-month-old CBA mice lost 69·12%. PE cells from 2–4-month-old C57 B1 mice lost 72·36% and those from 8–12-months lost 71·91%. However, within these two strains (CBA and C57 B1) the amount of isotope lost at 24 hr by the 2 age groups was not significantly different ($P > 0{\cdot}05$). But, as can be seen from Fig. 2, the younger age groups of the NZB/B1 and BALB/c strains lost the isotope-labelled antigen more rapidly in the earlier stages than do the older groups. By 8 hr (in the case of the NZB/B1 mice) and by 24 hr (in the case of the BALB/c mice), this difference was no longer significant.

(b) *The percentage of $I^{125}$-BSA lost by the peritoneal exudate cells to the $I^{125}$-BSA-free medium over 24 hr, following pulse labelling with $I^{125}$-BSA for 16 hr, which has been degraded by the PE cells (i.e. which is not precipitable by 20% TCA)* (Ehrenreich & Cohn, 1967)

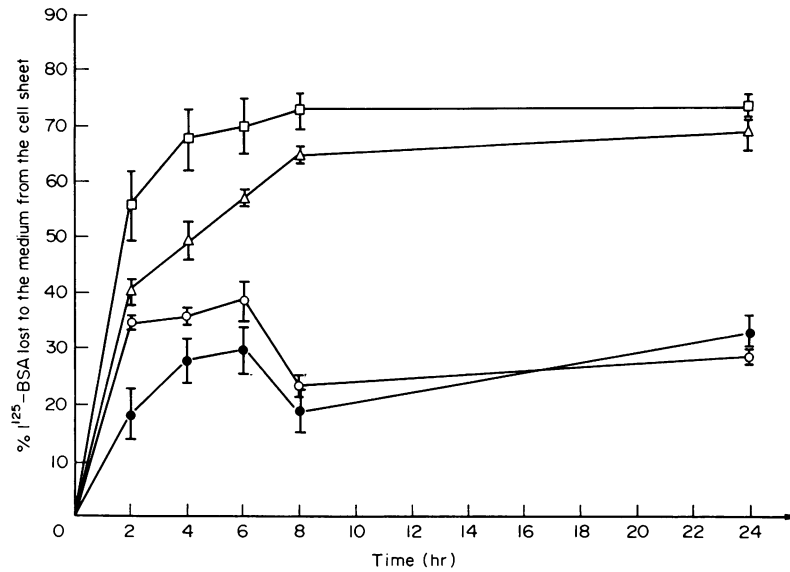


FIG. 2. The loss and degradation of $I^{125}$-BSA by peritoneal exudate cells. The percentage of $I^{125}$-BSA originally taken up by peritoneal exudate cells over 16 hr in $I^{125}$-BSA medium, subsequently lost on incubation in $I^{125}$-BSA-free medium. Standard deviations included. Source of peritoneal exudate cells: (○) C−ve NZB/B1 aged 2–4 months. (□) BALB/c aged 2–4 months. (●) C+ve NZB/B1 aged 13–16 months. (△) BALB/c aged 13–16 months.

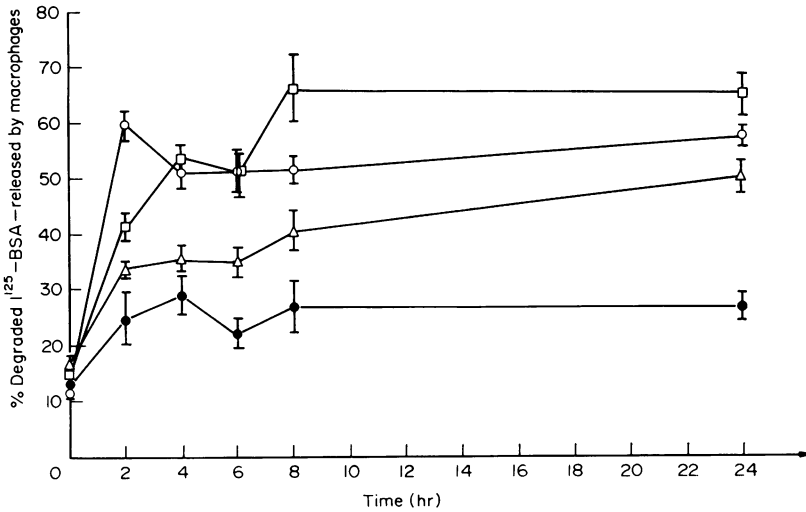


FIG. 3. The loss and degradation of $I^{125}$-BSA by peritoneal exudate cells. The percentage of $I^{125}$-BSA released by the peritoneal exudate cells during incubation in $I^{125}$-BSA free medium, following 16-hr pulse labelling with $I^{125}$-BSA, which has been degraded by the cells (i.e. not precipitated by 20% TCA). Source of peritoneal exudate cells: (○) C−ve NZB/B1 aged 2–4 months. (□) BALB/c aged 2–4 months. (●) C+ve NZB/B1 aged 13–16 months. (△) BALB/c aged 13–16 months.

There were highly significant differences between the activities of the cells from younger mice and those from older animals within each strain. In each case, the PE cells from the younger mice were more active in degrading the $I^{125}$-BSA (see Fig. 3). The BALB/c, and the CBA and C57 B1 (not shown in Fig. 3) strains all behaved in a similar manner giving results which varied significantly only in the differences between the younger and older age groups. After 24 hr incubation in $I^{125}$-BSA free medium 68·1% of the $I^{125}$-BSA lost to the medium had been degraded by the PE cells of CBA mice aged 2–4 months. This was reduced to 53·2% in CBA mice of 10–12 months of age. The C57 B1 mouse PE cells degraded 69·5% of the $I^{125}$-BSA when they came from mice aged 2–4 months, and 54·1% when they came from mice aged 8–12 months. Thus the younger mice of these three strains all degraded the $I^{125}$-BSA to roughly the same extent and this was to a greater extent than did the older mice of all three strains.

Initially the PE cells from NZB/B1 mice aged 2–4 months degraded more material than the age-matched BALB/c group; however, the difference was no longer significant at 4 and 6 hr incubation. At 8 hr and beyond, the activities were reversed, the BALB/c (and the CBA and C57 B1) PE cells being significantly more active that those of the young NZB/B1 group. The older (13–16-month-old) NZB/B1 mice gave very low (see Fig. 3) degradation results over the 24-hr incubation period.

## DISCUSSION

In this paper we have attempted to examine the *in vitro* activity of the peritoneal exudate cells of an auto-immune strain (NZB/B1) of mice and to compare this with a non-auto-immune strain (BALB/c), with a view to finding out if there are any differences in the way the strains take up and handle antigen. This might provide some insight into the apparent dysfunction of the lymphoid tissues of the NZB/B1 strain. In a subsequent paper we examine the interaction of lymphocytic and PE cells in the presence of antigen. In all of the experiments, the cells examined were those from the peritoneal exudate adherent to the glass coverslip after 1 hr incubation at 37°C.

In general, the uptake of $I^{125}$-BSA by peritoneal exudate cells of older mice, irrespective of strain was greater than that observed in younger animals. The PE cells of older NZB/B1 mice were particularly active at all stages of incubation in comparison with other strains as were young cells from young NZB/B1 mice during the first 8 hr of incubation. In contrast the PE cells of the younger mice were capable of degrading antigen to a greater extent than the PE cells from the older mice of the same strain (see Fig. 3). These findings could help to explain the results obtained by several authors using the NZB/B1 strain.

Playfair (1968) and Evans, Williams & Irvine (1968) observed the unusually early development of immune competence in these mice in their response to sheep red blood cells (SRBC). Near-adult levels of antibody were found in 5–7-day-old NZB/B1 mice. This behaviour was found to be similar in 6–18-week-old NZB/B1 mice (Morton & Siegel, 1969; Morton, Olson & Siegel, 1967; Siegel & Morton, 1967).

Other antigens, for example egg albumin and bovine gamma globulin were found to elicit a high antibody response in these mice (Staples & Talal, 1969). Weir, McBride & Naysmith (1968) showed an increased response in NZB/B1 mice to bovine serum albumin (BSA) and further commented on the difficulty in inducing a low zone tolerance to the same antigen. Playfair (1971) has recently been able to induce high zone tolerance to SRBC

in the NZB/B1 strain, where he had previously failed. This was achieved by greatly increasing the number of erythrocytes injected.

As the NZB/B1 animals age, in contrast to other strains, they show depressed primary antibody formation to SRBC immunization (Morton & Siegel, 1969; Diener, 1966; Morton & Siegel, 1968). The response of plaque-forming cells from the spleen of old NZB/B1 mice was found to be much lower than of age-matched BALB/c mice (Siegel, Brooks & Morton, 1970). Furthermore, whereas old BALB/c mice gave higher responses than young BALB/c mice, the situation was reversed with the NZB/B1 mice. The younger ones in this case gave higher plaque-forming responses than the old mice and were in fact more responsive than the old BALB/c mice.

The results of this study can be interpreted in terms of current views on tolerance induction and antibody formation, *viz* antigen being presented to lymphocytes after macrophage processing or with the aid of another helper cell leading to antibody formation, whereas direct contact between antigen and lymphocytes can result in immune tolerance, particularly after large doses of antigen.

Thus it would appear that when the NZB/B1 mouse is young, much of the antigen is taken up rapidly by the macrophages. It is therefore probable that insufficient passes directly to the lymphocytes to induce tolerance. Sufficient antigen, however, would be available to stimulate lymphocytes for antibody production.

As the NZB/B1 mouse ages, so more antigen is taken up by the macrophages and still less of this antigen is degraded. The consequences of this would be that in the older animal even less antigen would find its way directly to the lymphocyte to cause immune paralysis. Due to the markedly decreased degradation of antigen by the macrophages, less degraded, or processed antigenic material is available for transfer to lymphocytes (Fishman, 1961; Fishman & Adler, 1963; Fishman, Hammerstrom & Bond, 1963; Askonas & Rhodes, 1965; Cohen, 1967) and this could account for the decreased antibody plaque-forming potential observed in old, overtly autoimmune mice of this strain.

The implications of these observations to the state of autoimmunity in the NZB/B1 strain, is, of necessity, strictly hypothetical.

Burnet (1969) has defined autoimmunity as a condition in which structural or functional damage is produced by the reaction of immunocytes or antibodies with normal components of the body. In the normal individual there must be some form of control which enables the animal's immune system to recognize and not destroy its own tissues and organs. The maintenance of normal immune reactivity must depend on a delicately balanced interrelationship between the different cells of the lymphoid tissues resulting in the suppression or elimination of potentially self-reactive cells. The role of macrophages in such a process is likely to be of considerable importance.

The rapid and thorough removal of any antigen (self or non-self) from the circulation by hyperactive macrophages, as described here, might well enable any self-reactive clones in the NZB/B1 strain to escape from suppression or elimination and so produce anti-self antibodies. Such a process might be assisted by deficient T cell function in the NZB/B1 strain, as envisaged by Playfair (1971). One of the features of the NZB/B1 strain is the development of an autoimmune haemolytic anaemia and consistent with the view above is the finding that the peritoneal exudate cells from both C−ve and C+ve NZB/B1 mice are significantly more active in the uptake of erythrocytes from both C−ve and C+ve NZB/B1 mice as well as from other strains, than are the peritoneal exudate cells of the other strains themselves.

(Thomas, unpublished observations).

It is not possible from the present data to distinguish between an inherent characteristic of PE cells or extraneous environmental influences as the cause of the peculiar behaviour of such cells. This problem has been approached in a subsequent paper describing the role of lymphocytes in determining the activity of PE cells.

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