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Spontaneous antilymphoma reaction of preleukaemic AKR mice is a non-T-cell killing

THE high incidence of spontaneous leukaemias in AKR mice may be explained by the early appearance and high level of replication of a type C virus. It is often suggested, however, that in addition, AKR mice could be tolerant to their leukaemias. This tolerance would enhance the *in vivo* growth of malignant cells. In fact, AKR mice are not completely devoid of antileukaemic immune response[1–5]. It would be interesting, therefore, to determine if a quantitative or qualitative defect of one given type of antileukaemic response exists in AKR mice. Previous work has shown that an antibody response directed against the type C virus or against antigens of leukaemic cells can be demonstrated not only in hyperimmune[1] but also in normal AKR mice[4]. A cell mediated reaction directed against G (Gross)[6] leukaemia antigen-bearing cells was also detected *in vitro*[5,7] but this cell-mediated reaction was characterised by inhibition of the growth of G+ cells in monolayers. It is now known, however, that this kind of inhibition can be due to non-T cells[7–9] and could be antibody dependent[10]. A completely different cell-mediated antitumour reaction can be evidenced in the murine sarcoma virus (MSV) induced tumour system by the chromium release test (CRT) which detects a pure T-cell-mediated[11,12] and antibody-independent[13] cytolysis of tumour cells. Our work was performed to determine if AKR mice are capable of spontaneously developing such a kind of T-killer cell response.

AKR, C_3H/He, BALB/c and C57BL/6 mice were from our own colony. They have been tested at various ages from 2 weeks to 19 months without any preliminary treatment. Both males and females were used and tested individually. The CRT was done as previously described[13]. The effector cells were spleen cells, and the tumour target cells were lymphoma cells in suspension. A 100: 1 effector: target cell ratio was used. The serologically defined tumour antigens and the origin of the target cells are given in Table 1. The specific chromium release was measured after 18 h incubation of target cells in the presence of effector cells. It was expressed as the excess of release when target cells were incubated with AKR spleen cells in place of C_3H/He, BALB/c or C57BL/6 control cells. No difference in the chromium release can be detected when non-AKR cells were used as effector, whatever the age of the donor mice. As previously discussed, an excess of 4% in the chromium release can be considered as significant[13].

Results of experiments in which the effectors were whole spleen cells are given in Table 1. Individual AKR mice less than 3 months old were constantly negative. Positive CRT was found in 75% of 3–5-month-old AKR mice. The reaction was generally weak (specific release average = 12%), but could occasionally reach a high level (20–30%). Not only syngeneic or allogeneic G+FMRGi− lymphoma cells, but also G−FMRGi+ lymphoma cells could be lysed by the AKR spleen cells. Such a cross reaction was previously observed in MSV tumour system[13,14]. ERLD lymphoma and P815 mastocytoma, which bear no serologically defined leukaemic antigen[5], were never lysed by AKR spleen cells. Leukaemia of AKR mice began to appear after 5 month. Positive CRT reactions were observed in 41% of non-leukaemic animals older than 5 month. These results show that positive reactions are less frequent than in younger mice. In addition, positively-reacting animals were more frequent between 5 and 8 month (57%) than after 8 month (30%). CRT reactions were always negative in mice with overt leukaemia (enlargement of spleen, lymph nodes and thymus).

Table 1 Frequency of spontaneous reactions of AKR in CRT

Effector spleen cells	(G+) target cells*				(G−) target cells*		
	GL 3 (G+FMRGI−)	E♂G2 (G+FMRGi−)	SK 1 (G+FMRGi−)	Total of experiment with (G+) target	GiL 4 (G−FMRGi+)	ERLD (G−FMRGi−)	P 815 (G−FMRGi−)
2 weeks to 3 months old	0/32†	0/6	0/12	0/40	0/6	0/7	0/5
3–5 months old	26/35	3/4	4/5	33/44	6/10	0/3	0/7
Non-leukaemic: older than 5 months	21/46	1/4	5/13	27/63	3/12	0/23	0/3
Leukaemic: older than 5 months	0/14	0/2	ND‡—	0/16	0/4	0/7	ND

*GL 3 and E♂G2 = C57BL/6 Gross virus-induced lymphoma.
SK 1 = AKR spontaneous lymphoma.
GiL 4 = C57BL/6 Graffi virus induced lymphoma.
ERLD = C57BL/6 X-ray induced lymphoma.
P 815 = DBA/2 methyl-cholanthrene induced mastocytoma.
†Number of mice with positive results/number of tested mice.
‡ND = Not done.

Table 2 Effect of anti-θ AKR serum and complement treatment on the activity of spontaneously cytotoxic AKR spleen cells for the GL 3 lymphoma of C57BL/6 mice

Spleen cells from AKR	Specific chromium release (%) in the presence of reactive spleen cells treated by:				
	Medium alone	C_3H normal serum with complement	C_3H normal serum without complement	C_3H anti-θ AKR serum with complement	C_3H anti-θ AKR serum without complement
4 months old	25	23	22	27	28
5 months old	19	17	20	22	21
6 months old	11	12	9	13	12
7 months old	8	7	8	10	7
8 months old	15	12	13	14	13

These results suggest that AKR mice can develop a spontaneous antileukaemic reaction which appears during the preleukaemic period and completely disappears with the progression of the disease. It was previously shown that the CRT allows the detection of a pure T-killer cell phenomenon in our MSV tumour system[11,12] as well as during allograft rejection[15,16]. Thus, we have attempted to discover whether T cells could be responsible for the spontaneous CRT reactions detected in AKR mice. The whole spleen cell suspensions of individual AKR mice were treated with C_3H/He anti-θ AKR serum diluted at 1:10 and rabbit complement, before they were used in the CRT. Anti-θ AKR serum was obtained by repeated inoculations of AKR thymocytes in C_3H/He mice, the serum was absorbed by C_3H/He thymocytes and tested for its specificity for various lymphoid organs of AKR mice. Controls included in each experiments were first, AKR spleen cells treated by normal C_3H/He serum and rabbit complement and, second, AKR spleen cells treated by anti-θ AKR serum without complement.

The results of a typical experiment are summarised in Table 2. They show that the elimination of θ antigen-bearing cells does not decrease the antileukaemic response of AKR spleen cells. Twenty-three positive mice were individually studied giving identical results. It is unlikely that these results would be a consequence of a weak activity of the anti-θ AKR serum used, since this serum lyses all T-cells of AKR mice at dilutions up to 1:100 and because AKR receiving 4×10^7 C57BL/6 spleen cells intraperitaneally have cytotoxic lymphocytes in CRT for C57BL/6 target cells and pretreatment of these cytolytic cells with anti-θ AKR serum diluted at 1:10 and rabbit complement abolish their activity (Table 3). In addition, this last result demonstrates clearly that AKR mice are perfectly able to develop a T-killer cell response against an allogeneic graft. It can be concluded that the positive results obtained in the CRT with AKR spleen cells were due to non-T cells.

To study the nature of the effector cells, spleen cell suspensions from individual AKR mice were treated according to one of the following procedures (Table 4): first, incubation for 30 min at 37° C with carbonyl iron followed by six successive passages over a powerful magnet[16] This procedure removes practically all the macrophages. Second, filtration through anti-immunoglomulin-coated glass bead columns according to the method of Wigzell *et al.*[17]. This method removes both the macrophages and the immunoglobulin-bearing lymphocytes, and the eluted suspensions contain about 90% T cells. Third, incubation for 20 min at 37° C with 0.25% trypsin. The reaction was stopped by the addition of five volumes medium containing 10% foetal calf serum. This method allows the eventual removal of antigen-antibody complexes attached to effector cells. The results (Table 4) show that column filtration strongly decreases the CRT activity of spleen cells, and carbonyl iron treatment abolishes it completely. Identical results were obtained with the 18 reactive mice tested. It can be concluded that non-T cells, probably macrophages, are the main effector cells of the spontaneous CRT reactions of preleukaemic AKR mice. Treatment of spleen cells with trypsin suppress the CRT activity (Table 4), and this activity does not reappear even 24 h after trypsinisation. It is likely, therefore, that an antibody-dependent cell-mediated reaction is involved. We are studying the capacity of various AKR sera to block reactive cells and to 'arm' nonreactive cells in CRT.

Table 3 Effect of anti-θ AKR serum and complement treatment on the activity of anti-C57BL/6 immune AKR spleen cells for the GL 3 lymphoma of C57BL/6 mice

Anti C57BL/6 immune spleen cells from AKR	Specific chromium release (%) in the presence of immune spleen cells treated by:				
	Medium alone	C_3H normal serum with complement	C_3H normal serum without complement	C_3H anti-θ AKR serum with complement	C_3H anti-θ AKR serum without complement
2 months old	31	38	35	0	33
5 months old	23	26	24	0	27
8 months old	36	37	34	0	38

Three points should be noted. First, an antileukaemic reaction can be detected by the CRT in preleukaemic AKR mice, but this reaction, probably antibody mediated, is unable to protect the mice since it was found in at least 75% of 3–5-month-old AKR mice, whereas the incidence

Table 4 Effect of various treatments on the cytotoxic activity of reactive AKR spleen cells

Spleen cells from AKR	Specific chromium release (%) in the presence of reactive spleen cells treated by:			
	Medium alone	Column filtration	Carbonyl iron and magnet	Trypsin
4 months old	11	0	0	0
4 months old	21	7	0	0
5 months old	16	4	0	ND
7 months old	12	0	0	0
7 months old	9	0	ND	0
8 months old	8	0	0	0

of leukaemia in AKR mice is about 80%.

Second, we have demonstrated[11,12] in the MSV tumour system that the CRT allows the detection of pure T-cell killing of syngeneic tumour cells. Similar observations were done previously in allogeneic immunisation[15–16]. In the same experimental conditions, however, the AKR spontaneous reaction is caused by non-T cells. Immune cytolysis of P 815 tumour cells resulting from non-T cells have been also detected in old NZB mice[18]. Furthermore, it is known that in the microtoxicity assay (MA) both T and non-T cells[7,8] and even macrophages[9] can be involved. It therefore seems necessary to determine precisely the nature of the effector cells for each tumour system, whatever the *in vitro* methods used to study the cell-mediated immunity.

Third, the absence of T-killing in the antileukaemic response in preleukaemic AKR mice must be emphasised since the same animals can respond normally to allogeneic grafts with a high level of specific T-killer cells. The existence of such a reaction not only in young AKR but also in 8-month-old mice is especially interesting and suggests that a decrease with aging in the immune competence of AKR is not involved. It is noteworthy that the same AKR mice which develop anti-allogeneic T-killer cells can have at the same time a non-T cell mediated reaction directed against syngeneic G+ lymphoma. The first reaction is abolished by anti-θ AKR whereas the second remains unchanged. Preliminary results indicate that the absence of T-killer response is not a general property of the leukaemia viruses since resistant mice such as C57BL/6 are able to develop a 'T' response after Friend or Moloney virus inoculation (unpublished data). Further experiments in the Gross system using both sensitive and resistant mice are now in progress to determine whether the absence of T-killer cell response is a property of AKR mice or not. A hypothesis would be that the *Rgv-1* gene[19] which conditions the sensibility to Gross virus induced leukaemias could be responsible for the AKR inability to develop an antileukaemic T-killer cell response. This hypothesis could be reinforced by the fact that an H-2-linked gene, possibly identical to *Rgv-1* mapped in the H-2 complex near the 'Ir region'[20] has been recently shown to act like an immune response control gene[21].

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Observations on the mechanism by which T-lymphocytes exert cytotoxic effects

THE mechanism by which T lymphocytes kill tumour cells is unknown[1]. The killing is more rapid than reported for lymphotoxin[2]. Among possible mechanisms are the involvement of complement components[3] or phospholipase A, which could generate lysolecithin in the target cell membrane. To test these possibilities we have examined the rates of release from tumour cells of markers of high and low molecular weight and the effects of nonpenetrating solutes. If killing involves disruption of the structure of the cell membrane, for example by lysolecithin, simultaneous release of markers and no protection by macromolecular solutes would be expected. If lysis is osmotic, markers of low molecular weight should be released before those of high molecular weight and nonpenetrating solutes should protect against the lysis by counterbalancing the intracellular osmotic pressure. In the case of complement lysis, small macromolecules of molecular weight less than 40,000, which

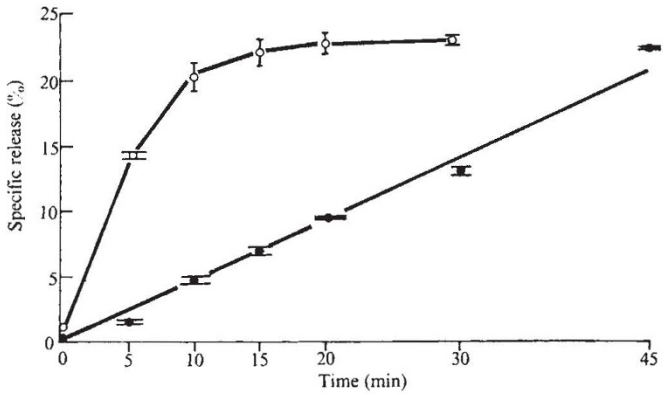

Fig. 1 Time course of the specific release of ^{86}Rb (O—O) and ^{51}Cr (●—●) from mastocytoma cells by sensitised spleen cells. P-815-X2 mastocytoma cells were double labelled: 5×10^6 cells were incubated for 1 h at 37° C with 100 μCi of $Na_2{}^{51}CrO_4$ and 1000 μCi of $^{86}RbCl$ in 1 ml Eagle's minimal essential medium containing 10% foetal bovine serum. Sensitised spleen cells were obtained from C57BL/6 mice injected intraperitoneally 11 d earlier with 3×10^7 live mastocytoma cells. Mixtures containing 10^7 normal or sensitised spleen cells and 1.5×10^5 labelled mastocytoma cells in 1 ml of the above medium were centrifuged in flat bottomed plastic tubes of 1.25 cm diameter and incubated at 37° C for the indicated lengths of time. Then they were mixed, briefly centrifuged and aliquots of supernatants taken for counting the released labels. Percentage specific release of the two labels was obtained by subtracting the release in the presence of normal spleen cells from the release in the presence of sensitised cells and is expressed as a fraction of the total labels released by freezing and thawing the cells. The values are means of duplicates and the range of variation is indicated.