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Lack of Correlation of Onset of Lymphomas and Levels of Murine Leukaemia Virus in (AKR × CBA/H-T6Crc) F1

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Abstract—*There is some evidence that the incidence of lymphomas in AKR hybrids parallels levels of murine leukaemia virus (MuLV). We originally noted that tumours were much delayed in the (AKR × CBA/H-T6Crc) F1 in spite of high levels of MuLV. The study of this cross has been extended to investigate any association between the level of MuLV with the incidence of lymphomas, any possible influence of maternal factors, and also to learn whether the delay of onset of lymphoma in the F1 is associated with the lack of anti-MuLV antibody activity.*

Results showed that although lymphomas were seen in reciprocal (AKR × CBA/H-T6Crc) F1 their occurrence was delayed. In contrast to the AKR, where tumours generally develop in the first year, tumours in the F1 occur in the second year of life. There was no difference in tumour incidence between the reciprocal normally derived crosses and crosses obtained following early embryo exchange transplantation. Levels of MuLV in spleen extracts of the F1 tested from 27 weeks onwards were, in general, higher than the parental AKR. However, this was only statistically significant in the (CBA/H-T6Crc × AKR) cross. There was also a higher level of MuLV in this cross when compared with the reciprocal (AKR × CBA/H-T6Crc) F1, although this was not statistically significant.

The trend towards a higher level of MuLV associated p30 antigen in the (CBA/H-T6Crc × AKR) F1 was also seen in the reciprocally embryo transplanted progeny, indicating no apparent maternal effect.

Finally, and in contrast to CBA/H-T6Crc immunized with Gross AKR virus, no anti-AKR MuLV activity was detected in the sera of the (AKR × CBA/H-T6Crc)F1.

INTRODUCTION

OUR INITIAL interest in the (AKR × CBA/H-T6Crc) F1 followed findings in a group of early embryo aggregation derived AKR↔CBA/H-T6Crc chimaeras. In the chimaeras, lymphomas were not only delayed [1] but also the overall incidence was reduced [2]. This observation was especially interesting when it was found that in spite of "balanced" coat colour composition and distribution of the gametes, each chimaera was essentially AKR when analysed cytogenetically [3, 4] or by cell product markers [5]. Relative tumour "resistance" in this situation had to be attributed to the relatively minor CBA cell component or cell product in the chimaeras. In subsequent investigations, the lack of detectable "free" anti-murine leukaemia virus (MuLV) antibody led us to suggest the possible association between tumour "resistance" and lack of anti-MuLV activity [6].

It was the observations mentioned above which prompted us to investigate the situation in the naturally derived (AKR × CBA/H-T6Crc) F1. Our preliminary findings confirmed the presence of MuLV—moreover, levels comparable with AKR [7]. In spite of this, tumours initially appeared uncommon during the first year. In retrospect high levels of MuLV was not surprising since the CBA/H-T6Crc like the AKR was also Fv-1^n and hence the F1 Fv-$1^{n \times n}$ might be anticipated to be permissive to N-tropic AKR MuLV infection [8].

The further examination of this particular

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hybrid seemed important (a) to confirm that the incidence of tumours was delayed in spite of high levels of MuLV (b) to investigate previously suggested maternal influence on both incidence of tumours and viral load and finally (c) to see whether,, like the chimaeras, anti-MuLV antibody activity was not demonstrable. The latter appeared especially important since we earlier suggested that the lack of such antibody activity at least in the chimaeras might be a factor influencing relative tumour "resistance".

MATERIALS AND METHODS

1. *Mice*

AKR/Crc (formerly AKR/J) and CBA/H-T6Crc (CBA in text) were used to provide reciprocal crosses and these were examined with AKR and CBA controls. The derivation of these sublines has been described elsewhere [9].

Various groups of mice were examined:

(a) The main group of normally derived mice was studied to determine the incidence of tumours. When signs suggestive of tumour development occurred, or occasionally following natural death, post mortem was performed and evidence of lymphoma sought macroscopically. On occasions, when there was any doubt, histology was performed upon haemtoxylin and eosin stained sections.

(b) A second group of normally derived mice were electively sacrificed from 27 weeks of age onwards for determination of p30 levels. These animals were not macroscopically lymphomatous.

(c) A third group of reciprocal crosses were derived following early embryo exchange transplantation, a technique described in full elsewhere [10]. In practice this meant that (AKR ♀ × CBA ♂) F1 were transplanted and born from CBA mothers and in reverse (CBA ♀ × AKR ♂) F1 transplanted and born from AKR mothers. These animals were followed for incidence of tumours and levels of p30 were determined at death when lymphomas were apparent.

(d) A different group of naturally derived hybrids were tail bled for serum samples for natural anti-MuLV activity during the first year of life.

(e) Finally, a group of CBA was injected intraperitoneally at 6 wk of age with 0.1 ml Gross passage A virus (1.2×10^4 F.F.U/ml). These were bled 6 weeks later for evidence of anti-MuLV activity.

2. *Viral antigen screening*

Radioimmunoassay was performed upon splenic homogenates to measure levels of MuLV associated group specific antigen p30. The radioimmunoassay was a modification of the original method described by Strand and his co-workers [11]. In practice 6 μg Rauscher p30 antigen (kindly supplied through the courtesy of Dr. J. G. Gruber at N.C.I.) was radio-labelled with ^{125}I to achieve a specific activity of about 18 μCi/μg using the chloramine T method [12]. The radio-labelled antigen was subsequently divided into aliquots, snap frozen in liquid N_2 and then stored at −35°C.

The primary reaction consisted of incubating 50 μl of a dilution goat anti-AKR with 100 μl of the tissue extract at 37°C for 18 hr. The dilution of the antisera used was known to precipitate approx. 50% of radio-labelled antigen. In practice TEN buffer [20mM Tris–hydrochloride pH 7.6, 1 mM ethylenediamine tetracetate (EDTA) with 100 mM NaCl containing 0.2% Triton ×-100 made up in 20mg/ml crystalline BSA solution] was used to dilute the mixture to a total volume of 200 μl.

In the secondary reaction, precipitation of the primary antibody–antigen complex was achieved by adding 50 μl of a dilution of pig anti-goat IgG known to achieve maximum precipitation. Incubation at 37°C for 2 hr was subsequently followed by incubation overnight at 4°C. Half of a millilitre of TEN buffer (with CBSA-2 mg/ml) was then added and following centrifugation (1790g for 40 min) and careful removal of the supernatant, radioactivity of the precipitate was determined in an NE 160 automated gamma counter.

In each case splenic assay samples were examined in duplicate and levels of p30 were determined by extrapolation from a standard inhibition curve.

Values of p30 were expressed as ng/1.0 mg of tissue protein—the latter determined using Lowry's method [13].

3. *Anti-MuLV Assay*

Radioimmunoassay against [^{3}H] leucine labelled intact AKR virus was used to screen the serum samples for anti-MuLV activity. The technique is described in detail elsewhere [14] but in practice consisted of treating radio-labelled virus with 1:40 dilution of serum (37°C for 1 hr) and subsequent treatment with an optimal dilution of the secondary

heterologous precipitating anti-mouse globulin (37°C for 1 hr and at 4°C for 2 hr). Precipitation was subsequently expressed as the percentage of the counts in the precipitate relative to the combined counts in the precipitate *and* supernatant. As mentioned earlier the technique is described in full elsewhere [14].

RESULTS

1. *Tumour incidence*

The incidence of lymphomas in the reciprocal AKR × CBA crosses together with the AKR controls is shown in Fig. 1. Some of this data has been presented earlier in a preliminary communication [7]. It is quite obvious from these data that compared with the parental AKR, lymphomas were delayed in both crosses (Fig. 1). Tumours appeared somewhat earlier in the (CBA × AKR) F1—mean age 79 weeks. In the reciprocal (AKR × CBA) F1 the mean age was 87.6 weeks but the lymphomas in the AKR appeared associated with a grossly enlarged thymus, whereas in the F1 it was not uncommon to find evidence of lymphomatous splenic and/or lymph node enlargement whilst the thymus appeared normal.

The incidence of tumours in the reciprocal crosses obtained following early embryo exchange transplantation and derivation from the corresponding but opposite mother is also shown in Fig. 1. The incidence quite clearly was no different from the corresponding naturally derived cross. These results rule out the possibility of any maternal effect upon the incidence of tumours.

2. *Levels of p30*

Results in the competition radioimmunoassay are summarised in Table 1 where

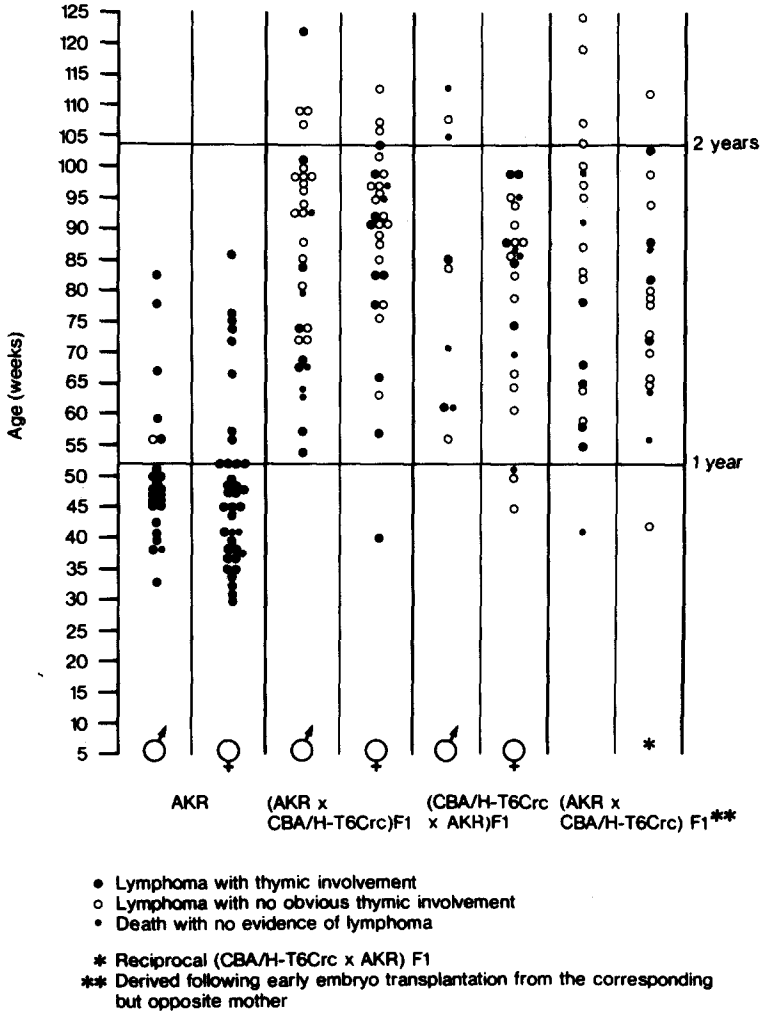

Fig. 1. Incidence of lymphomas in reciprocal (AKR × CBA/H-T6Crc) F1.

Table 1. Levels of the MuLV-Associated antigen p30 in spleens of (AKR × CBA) F_1 and controls*

Mice	No. tested	Age range (weeks)	Level of p30† Mean ± S.D.	Range
AKR	28	26–34	23.7 ± 6.9	12.8 ± 35.3
CBA/T6Crc	27	34–112	9.2 ± 4.2	1.9–18.2
(AKR ♀ × CBA ♂) F_1 (AKR ♀ × CBA ♂) F_1 embryo transplanted into CBA ♀	18	27–85	27.1 ± 11.0	14.1–55.2
	10	55–124 (at death)	21.5 ± 15.2	9.9–60.3
(CBA ♀ × AKR ♂) F_1 (CBA ♀ × AKR ♂) F_1	12	33–52	47.4 ± 34.2	4.5–129.7
embryo transplanted into AKR ♀	9	72–112 (at death)	39.2 ± 29.1	13.5 ± 109.7

* CBA/H-T6Crc
†ng of p30/mg tissue protein

it can be seen that levels of p30 covered a considerable range. Age differences and the limitations of the assay could have contributed to this. However, it can be seen that the p30 levels for the CBA controls were very much lower (9.2 ± 4.2) than the macroscopically normal AKR controls (23.7 ± 6.9). The levels in the macroscopically normal naturally derived reciprocal crosses covered a larger range. In both cases levels were higher than the AKR, but only in the (CBA × AKR) F1 (47.4 ± 34.2) was this significantly higher than the parental AKR ($P < 0.05$). Although levels in the (CBA × AKR) F1 appeared higher than those in the reciprocal (AKR × CBA) F1, the difference here was not statistically significant. The fact that this trend was confirmed in early embryo exchange transplanted hybrids born and subsequently milk fostered from the corresponding but opposite mother rules out the possibility of a maternal effect in respect of levels of the MuLV associated p30 antigen.

3. *Anti-MuLV-antibodies*

Results here were clear cut.

As can be seen from Fig. 2, the majority of the adult CBA injected with AKR virus showed an antibody response (> 30%) against the radio-labelled AKR virus in immunoassay. In contrast, no antibody activity (> 30%) could be demonstrated in any of the 75 individual serum samples obtained from the reciprocal (AKR × CBA) F1 (Fig. 3). Within the context of the known sensitivity of this assay, results suggest the absence of "free" anti-AKR MuLV activity in the F1 up to 1 yr of age when the last samples were obtained.

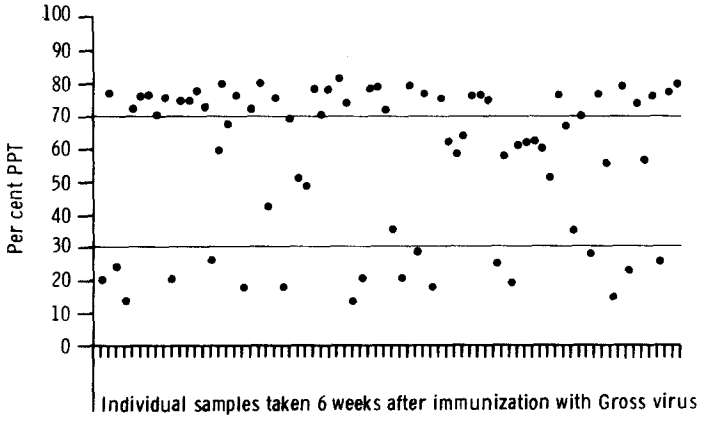

Fig. 2. Anti-MuLV activity in adult CBA/H-T6Crc mice immunized with Gross virus.

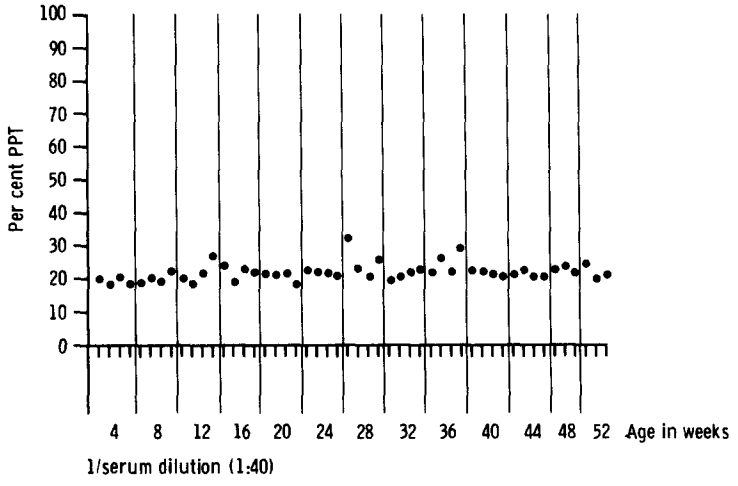

Fig. 3. Lack of detectable anti-MuLV activity in individual serum samples obtained from reciprocal (AKR × CBA/H-T6Crc).

DISCUSSION

From the findings here, it is quite clear that lymphona susceptibility is not invariably linked to the viral load in AKR derived hybrids. The delay in development of tumours in the F1 is, in the case of the AKR × CBA cross, independent of viral load. Although we have confined our investigation of virus here to examination of the group specific MuLV p30 antigen, previous studies showed that this was also seen in the (CBA × AKR) F1 transtropic MuLV [7]. The levels of p30 in the naturally derived F1 hybrids were higher than the AKR, although only significantly in the (CBA × AKR) cross. In spite of this, tumour development was generally confined to the second year of life. Although the high viral load might be anticipated since both AKR and CBA are Fv-1^n and hence permissive to N-tropic AKR MuLV infection [8] the fact both strains are also H-2^k and the F1 H-$2^{k \times k}$ makes the delay in onset of lymphoma development surprising since H-2^k status generally confers susceptibility to viral associated tumour development [15, 16].

Maternal influences are known to effect the incidence of lymphoma in AKR × low lymphoma strain crosses [17, 18]. However, no maternal effect was demonstrated here. No difference in the incidence of tumours was detected in the naturally derived reciprocal crosses. Furthermore, the same was true for the two groups of reciprocal crosses derived following early embryo exchange transplantation and being born and milk fostered upon the corresponding but opposite mothers. However, in respect of levels of p30, there did appear to be certain differences. Although in both naturally derived F1 hybrids, the levels of p30 appeared higher than the parental AKR, this difference was confined statistically to the (CBA × AKR) F1 and this tendency was also seen in the (CBA × AKR) F1 transplanted, born and milk fostered from the AKR. The significance of this is uncertain. Maternal influence is sertainly ruled out—heterosis is possible explanation, although it is quite clearly not related to tumour development where the incidence between all four groups of crosses appeared comparable.

Of course here we have examined the MuLV group specific associated antigen p30. Recently it has been suggested that a recombinant eco-xenotropic virus may be responsible for the lymphoma of the AKR [19] implying an essential role of xenotropic virus in spontaneous leukaemogenesis. The evidence, although circumstantial, is supported by Lilly's data with the AKR × Rf cross [20]. This cross is also low leukaemic which could be attributed to the fact that these animals rarely express xenotropic MuLV before about 14 months of age. It appears that this delay is governed by the presence of the Fv-1 allele of the Rf. The same may be true for the (STS × AKR) F1 [16] and perhaps the (CBA × AKR) F1 since neither the STS nor the CBA express xenotropic MuLV and this may be a decisive factor in crosses with AKR. Studies in expression of xenotropic MuLV are therefore in progress.

We earlier suggested that the lack of anti-AKR activity might be a factor influencing the relative tumour "resistance" in the AKR↔CBA chimaeras—a component contributed by the CBA involved in maintaining tolerance to the Gross virus [6]. It is now

known that mice of almost all inbred strains produce natural antibody to MuLV [21]. Although no natural MuLV has yet been detected in the CBA, they are clearly capable of mounting an immune response to injected AKR virus, although it must be remembered that the inoculum was with Gross-passaged A virus and conceivably antigenic differences to natural AKR virus might be responsible for the marked immune response in these animals.

In contrast, anti-MuLV activity was not detected in any of the 75 (AKR × CBA) F1 serum samples obtained during the first year of life. Of course, it could be argued that anti-MuLV antibody activity was present and the failure to demonstrate *detectable* antibody was due to the fact that all antibody was complexed to corresponding antigen. At this stage, we cannot exclude this possibility, although preliminary results make this seem unlikely. If we confirm the absence of anti-MuLV activity in the F1, we are led to question, as in the case of the AKR↔CBA chimaeras, how tolerance to MuLV is apparently maintained, furthermore, is the lack of detectable anti-MuLV activity and the delay in onset of lymphoma development in the F1 in anyway related. If this is so then anti-MuLV activity would be detected during the second year of life when tumours appear—a period, unfortunately, that has yet to be examined.

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REFERENCES

1. R. D. Barnes, M. Tuffrey and J. Kingman, The delay of leukaemia in tetraparental ovum fusion derived AKR chimaeras. *Clin. exp. Immun.* **12,** 541 (1972).
2. R. D. Barnes, M. Tuffrey and C. E. Ford, Suppression of lymphoma development in tetraparental AKR mouse chimaeras derived from ovum fusion. *Nature, New Biol.* **244,** 282 (1973).
3. M. Tuffrey, R. D. Barnes, E. P. Evans and C. E. Ford, Dominance of AKR lymphocytes in tetraparental AKR↔CBA-T6T6 chimaeras. *Nature, New Biol.* **243,** 207 (1973).
4. C. E. Ford, E. P. Evans, M. D. Burtenshaw, H. Clegg, R. D. Barnes and M. Tuffrey, Marker chromosome analysis of chimaeras: dominance of AKR mitoses in tetraparental AKR↔CBA-T6 mice. *Differentiation* **2,** 321 (1974).
5. R. D. Barnes, M. Tuffrey, L. Drury and D. Catty, Unequal rates of cell proliferation in tetraparental mouse chimaeras derived by fusion of early embryos. *Differentiation* **2,** 257 (1974).
6. R. D. Barnes, M. Tuffrey and R. C. Bourne, Failure to detect anti-group-specific murine leukemia virus activity in tetraparental AKR-CBA chimeras, *Cancer Res.* **35,** 2699 (1975).
7. R. D. Barnes, M. Tuffrey, P. R. Crewe, L. Dawson, K. Brown and J. Joyner, Levels of C-type viral p30 antigen in lymphoma-resistant mice. *Cancer Res.* **36,** 3622 (1976).
8. W. P. Rowe, Studies of genetic transmission of murine leukaemia virus by AKR mice I. Crosses with Fv-1^n strains of mice. *J. exp. Med.* **136,** 1272 (1972).
9. R. D. Barnes and M. Tuffrey, Absence of lymphomas in CBA mice derived by embryo transfer and born from lymphoma-prone AKR mice. *Europ. J. Cancer* **10,** 575 (1974).
10. R. D. Barnes, M. Tuffy, J. Kingman and R. A. Risdon, The disease of the NZB mouse: examination of exchange ovum transplantation derived NZB and CFW mice. *Clin. exp. Immunol.* **10,** 493 (1972).
11. M. Strand, F. Lilly and J. T. August, Host control of endogenous murine leukaemia virus gene expression: concentrations of viral proteins in high and low leukaemia mouse strains. *Proc. nat. Acad. Sci. (Wash.)* **71,** 3682 (1974).
12. W. M. Hunter, The preparation and assessment of iodinated antigens. In *Radioimmunoassay Methods: European Workshop.* (Edited by K. E. Kirkham and W. M. Hunter). p. 3. Churchill Livingstone, Edinburgh (1971).
13. O. H. Lowry, N. U. Rosebrough, A. L. Farr and R. J. Randall, Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265 (1951).

14. J. N. IHLE, M. YURCONIC, JR. and M. G. HANNA, JR., Antrogenous immunity to endogenous RNA tumour virus: radioimmune precipitation assay of mouse serum antibody levels. *J. exp. Med.* **138,** (1973).
15. H. MEIER, B. A. TAYLOR, M. CHERRY and R. J. HUEBNER, Host-gene control of type-C RNA tumour virus expression and tumorigenesis in inbred mice. *Proc. nat. Acad. Sci. (Wash.)* **70,** 1450 (1973).
16. F. LILLY, M. L. DURAN-REYNALS and W. P. ROWE, Correlation of early murine leukaemia virus titer and H-2 type and spontaneous leukaemia in mice of the BALB/c x AKR cross: a genetic analysis. *J. exp. Med.* **14,** 882 (1975).
17. L. W. LAW, Maternal influence in experimental leukaemia of mice. *Ann. N.Y. Acad. Sci.* **57,** 575 (1954).
18. J. FURTH, R. K. COLE and M. C. BOON, The effect of maternal influence upon spontaneous leukaemia of mice. *Cancer Res.* **2,** 280 (1942).
19. J. W. HARTLEY, N. K. WOLFORD, L. J. OLD and R. P. ROWE, New class of murine leukaemia virus associated with development of spontaneous lymphomas. *Proc. nat. Acad. Sci. (Wash.)* **74,** 789 (1977).
20. A. MAYER, ML. DURAN-REYNALS and F. LILLY, Fv-1 regulation of ecotropic and xenotropic MuLV expression in the thymus and lymphoma development in mice of the AKR/J x RF/J cross. *Cell* **15,** 429 (1978).
21. R. NOWINSKI and S. KAEHLER, Antibody to leukaemia virus—widespread occurrence in inbred mice. *Science* **185,** 869 (1974).