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Lymphoma Development in AKR:CBA/H-T6Crc Chimaeras Derived by Neonatal Injection of Spleen Cells

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Abstract—*Reciprocal neonatal injections of spleen cells into CBA/HT6Crc and AKR mice rendered these animals highly tolerant as judged by persistence of reciprocal skin grafts. Chimaerism was demonstrated in several cases, although there was no AKR "takeover" as demonstrated in previous studies with early embryo aggregation (E.E.A.) chimaeras. Lymphoma incidence in AKR mice made tolerant to CBA was similar to that seen in normal AKR. In contrast to normal CBA in which there is no incidence of lymphoma, the CBA made tolerant to AKR died with lymphomas. However, the latent period was delayed and was similar to that seen in (CBA × AKR) F1 hybrids. Murine leukaemia virus (MuLV) p30 levels in the tolerant CBA were also elevated and comparable to those seen in AKR. Reciprocal thymic grafting, which was in some groups of mice accompanied by further injections of lymphoid cells from the donor strain, appeared to have no effect on lymphoma incidence. The CBA lymphomas were, therefore, attributed to MuLV present in the original spleen cell injections. These findings differ from those of Miller, who found that there was no occurrence of lymphoma in low leukaemic C3H mice made tolerant of AK_1 cells during the neonatal period.*

INTRODUCTION

WE HAVE shown that the incidence of lymphomas in a group of AKR↔CBA/H-T6Crc chimaeras derived by early embryo aggregation (E.E.A.) was both delayed [1] and reduced [2] despite the fact that these animals had an overwhelming number of AKR cells [3, 4] cell products [5] and type C murine leukaemia virus particles [6] which are associated with the high incidence of lymphoma in the AKR strain of mice.

In these experiments we have tried to simulate E.E.A. chimaeras by making groups of AKR and CBA/H-T6Crc mice reciprocally tolerant of each other by injection of spleen cells during the first 24 hr of life. We have also attempted to study the influence of the thymus in this situation by means of thymic grafting.

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MATERIALS AND METHODS

Experimental animals

(i) *Induction of tolerance.* AKR/Crc and CBA/H-T6Crc (AKR and CBA in text) were made reciprocally tolerant by the intravenous injection of spleen cells during the first 24 hr of life.

Spleen cell suspensions were prepared from young mice. Spleens were removed aseptically and placed in a small amount of media (L15). They were minced with a pair of curved scissors then sieved. The resulting cell suspension was spun at 2000 rev/min for 5 min and the supernatant was decanted. The cells were then resuspended in fresh media and the suspension allowed to stand for about 3–4 minutes to allow larger cell clumps to settle. The supernatant was subsequently removed and centrifugation repeated. The cells were resuspended and a viable cell count was obtained using Trypan Blue. Spleen cells 4–5 $\times 10^6$ in 50 μl was injected via the anterior facial vein during the first 24 hr of life. Any respiratory distress was corrected by pressure on the abdomen—the injected neonates being

subsequently returned to their mothers after recovery. Tolerance was subsequently confirmed by skin grafting.

When the injected neonates were 6–8 weeks of age, they were grafted with tail skin of the same strain as the injected spleen cells. The grafts were dressed with Vaseline gauze and kept in place with Plaster of Paris bandages. Dressings were removed on day 7 and grafts were subsequently observed at regular intervals and survival observed macroscopically.

(ii) *Thymus grafting and additional lymphoid cell injections.* Approximately half of the neonatally injected animals were grafted at various ages into the axilla with a whole thymus taken from a month old donor of the same strain as the injected cells. The remaining animals were untreated.

In the case of the AKR, CBA thymuses were grafted when the "injection chimaeras" were 10–13 weeks of age. In order to increase the "chimaerism" in these thymus grafted animals, further CBA lymphoid cell suspensions of both spleen and thymus were given. Approximately 10×10^6 CBA spleen cells were injected I.P. between 10 and 16 weeks of age, and a further four separate injections of CBA thymus cell suspension, each prepared from one thymus, were given to the thymus grafted "chimaeras" when they were between 10 and 26 weeks of age.

In the case of the CBA neonates injected with AKR cells, the majority of these animals were grafted into the axilla with an intact thymus from a month old AKR donor at about 26 weeks. In a few cases a second thymus was implanted but none of these chimaeras was given additional lymphoid cell suspensions.

Investigations

(i) *Routine investigation.* All of the mice were routinely examined for persistence of skin grafts and for evidence of lymphoma development. They were sacrificed when they appeared moribund and pieces of lymphoid tissue, liver and kidney were taken for routine histology.

(ii) *Tests for chimaerism.* During life various tests were carried out to confirm chimaerism. The serum complement factor C'5 (Muβl) was assayed on early serum samples from the injected AKR mice, using an Ouchterlony technique with a specific anti-Muβl sera. The AKR strain are deficient in Muβl [7] whilst the CBA/H-T6Crc have normal levels of serum complement [8].

The strains also possess differing variants of the red cell isoenzyme glucose phosphate isomerase (g.p.i.) which can be distinguished by cellulose acetate electrophoresis and quantitated by means of an LKB "Microzone" computing desitometer [5]. Red cell lysates from whole blood were tested from the AKR mice whilst spleen extracts were assayed from the CBA group.

Since the CBA mice used here have a chromosomal marker (T6), blood and tissues from the chimaeras were occasionally examined cytogenetically for evidence of chimaerism.

Blood cultures were set up according to the method devised by M. D. Burtenshaw. In practice this involved collecting nine drops (0.2 ml) of tail blood in 5 ml of rinsing medium RPMI 1640 supplemented with 300 i.u./ml penicillin, 300 μg/ml streptomycin and 70 i.u./ml preservative free heparin. After centrifugation at 1200 ***g*** for 10 min, the cells were resuspended in the same volume of fresh medium. After a second centrifugation (1200 ***g***/10 min) the cells were resuspended in 5 ml of culture medium RPMI 1640 containing 4 mM L-glutamine, 300 i.u./ml penicillin, 300 μg/ml streptomycin, 20% foetal calf serum, 70 i.u./ml heparin and Concanavalin A at a final concentration of 10 μg/ml. Sterile disposable bijoux (Sterilin) were used to collect and culture the samples.

The cultures were incubated at 37°C for 4 days and harvested after the addition of Colcemid (or vinblastine sulphate) at a final concentration of 0.1 μg/ml for 2–4 hr. Centrifugation (300 ***g***/5 min) was followed by initial resuspension in 5 ml of serum-free medium and then subsequently (after further centrifugation) in 5 ml of 0.075 M KCl for 8 min. After centrifugation (150 ***g***/5 min) the cells were fixed in 3:1 methanol:acetic acid. The pellet was gently dispersed by flicking the centrifuge tube and 1 ml of fixative was added slowly. After 2 min the fixative was changed and the procedure then repeated 3 times, the final fixation being in 5 ml of fixative for 5 min. The tubes were then centrifuged (200 ***g***/5 min) and the cells resuspended in 1 ml fresh fixative. Slides were made by air drying, hydrolysed in 5 N HCl for 2 min and stained with toluidine blue resin [9].

Direct chromosome preparations were obtained from bone marrow, spleen, thymus, lymph nodes and liver by a standard air drying method [4] with minor modifications, which included the use of Vinblastine sulphate (0.1 ml of a 1 mg/ml solution injected intraperitoneally) as a mitotic arrestant in

place of Colcemid; and 0.07 M KCl (10 min) as the hypotonic treatment.

(iii) *Levels of viral antigen.* Spleen homogenates were screened for levels of the MuLV group specific antigen p30 using a radioimmunoassay. In each case the tissues were frozen individually at −35°C, subsequently thawed at room temperature then homogenised using a Potter Mill with approximately ×3 vol of cold (4°C) phosphate buffered saline (pH 7.4). The homogenate was subsequently removed and then stored at −35°C until used in the radioimmunoassay.

The radioimmunoassay for the group specific antigen p30 was a modification of the original method described to by August, Lilly and Strand [10]. In practice p30 antigen (kindly supplied through the courtesy of Dr. J. G. Gruber at N.C.I.) was radiolabelled with ^{125}I using the chloramine T method [11].

The primary reaction consisted of incubating 50 μl ^{125}I labelled antigen (1 ng per tube), 100 μl of the tissue extract with 50 μl of an optimum dilution goat anti-AKR p30 at 37°C for 18 hr.

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

In each case 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 [12].

RESULTS

Grafts of AKR or CBA tail skin onto the reciprocal recipients are normally rejected within 10–14 days. Of the 33 (20♀ 13♂) CBA "chimaeras" grafted with AKR tail skin, 30 animals had perfect grafts present at death (350–728 days). The rejections occurred on days 71, 86 and 117. Results are summarized in Table 1.

Fifty seven (28♂; 29♀) AKR "injection chimaeras" were grafted with CBA tail skin. Grafts persisted until death in only 9 of the 28 AKR males (217–466 days). The remaining 19 animals (68%) rejected their grafts, usually between 108 and 218 days, although one rejected its graft much earlier (between days 57 and 68) and another much later (at about day 500). Eight of the 29 female AKR had grafts present at death between day 175–252. The remaining 21 females (72%) rejected their grafts between 100 and 200 days. Results are again summarized in Table 1.

It should be noted that rejections were slow and difficult to assess precisely, macroscopically. Thus the rejection times were calculated as being the mean of the total time considered to be taken for grafts to be rejected. This was frequently over a period of up to 3 weeks. Histological assessment of the grafts was made when possible.

From the results it is quite clear that these animals were tolerant although the CBA animals showed the more permanent tolerance as judged by acceptance of skin grafts.

In spite of persistence of skin grafts, it was frequently difficult to detect other criteria of chimaerism. Muβl was not detected in any of the AKR "injection chimaeras" nor was the

Table 1. *Reciprocal strain skin graft survival and cause of death in tolerant CBA and AKR mice*

Strain	No. of animals	No. with skin graft at death (age at death—range) (days)	No. of animals rejecting grafts (ages at rejection—range) (days)	No. of animals with lymphoma
CBA/T6♂	13	12 (448–686 d)	1 (86)	13
CBA/T6♀	20	18 (350–728)	2 (71, 117)	14
AKR♂	28	9 (217–466)	19 (108–218) mean 162 S.E. 86.7	26
AKR♀	29	8 (175–252)	21 (100–200) mean 128 S.E. 22.1	27

Table 2. Cytogenetic analysis of AKR: CBA/H T6Crc "injection chimaeras"

	No. of mitoses scored					
	Blood	Thymus	Spleen	Bone marrow	Lymph node	Liver
AKR	1624	235	560*	432	143	46
CBA/T6	n.t.	152	1250†	50	350	50

*6 T6T6 foreign cells (in 4/12 animals).
†96 AKR foreign cells (in 5/22 animals).

g.p.i. variant associated with the CBA. However, of the 26 spleen extracts from the CBA "chimaeras" tested for g.p.i., 7 animals showed AKR variant levels ranging from 13 to 30%.

Chimaerism was also demonstrated *cytogenetically* in some cases. Tissues from 12 of the AKR "chimaeras" were tested for the presence of CBA/H-T6T6 cells. These included spleen, thymus, liver, bone marrow, lymph node and blood culture. Four of the 12 animals had evidence of chimaerism ranging from 2 to 4.7% T6T6 cells. These foreign cells were only found in spleen preparations, although many mitoses from all the tissues were examined (Table 2).

A similar range of tissues was tested from the CBA "chimaeras". Of the 22 animals tested (1852 cells scored) 5 were found to have AKR cells, again only in their spleens, ranging from 2 to 100% (2, 9, 10, 50 and 100%). It was interesting to note that the lymphomatous CBA whose spleen showed 100% AKR mitoses had a lymphomatous mesenteric lymph node which was 100% T6T6.

The incidence of lymphoma in both types of "chimaeras" is shown in Fig. 1 together with controls. The AKR animals injected with CBA spleen cells showed an incidence of lymphomas comparable to normal AKR and also commonly presented with thymic enlargement. The incidence of lymphoma in the CBA neonatally injected with AKR spleen cells was similar to that seen in (AKR × CBA) Fl and in both cases thymic enlargement was less frequent.

It is interesting to note that neither thymus grafting nor additional injections of lymphoid cells appeared to have any effect on the lymphoma incidence in these mice. Difficulty was experienced in retrieving these grafts and no dividing cells suitable for cytogenetic analysis were found in any of the 6 grafts obtained at sacrifice from the AKR. Of the 6 thymus grafts of AKR origin retrieved after transplantation into the CBA mice, two were found to have 100% T6T6 mitoses and one of these was lymphomatous. A further two mice had lymphomatous infiltrations involving the graft but cytogenetic analysis was not possible. The lymphomas in the tolerant CBA were host in origin.

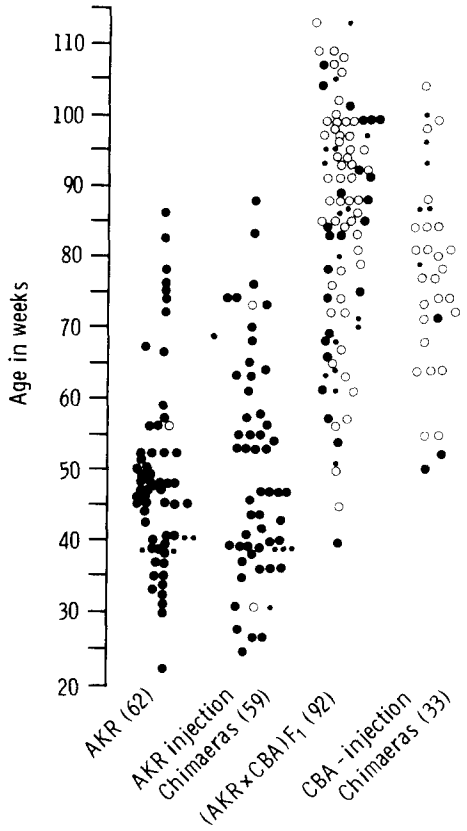

Fig. 1. Incidence of lymphomas in control and experimental mice. Lymphoma with thymic enlargement (●), lymphoma with no thymic enlargement (○), no lymphoma found (·).

The mean levels of p30 in the spleen homogenates expressed as ng/mg tissue protein are shown below.

AKR	29.01 ± 7.5
CBA	5.96 ± 0.49
CBA*	34.64 ± 5.33

*Injected with AKR cells

From these results it can be seen that the p30 levels in the lymphomatous CBA mice injected with AKR cells at birth, were comparable to the levels seen in untreated AKR controls and significantly higher than untreated CBA controls [13].

DISCUSSION

Results were clear cut both as to the induction of tolerance and the incidence of tumours. It has been well established that the intravenous injection of spleen cells between H-2 compatible strains during the newborn period provides the best situation for induction of a high degree of tolerance [13]. it is also known that the duration of tolerance can be variable ("asymmetrical") when induced reciprocally between two strains [13]. In this experiment tolerance was induced reciprocally between the AKR and the CBA (both H-2^k) but was maintained much longer in the latter.

Billingham and Brent state that "there can be no doubt that the systemic inoculation of newborn mice with homologous cell suspensions leads to the formation of cellular chimaeras" [13]. Although they demonstrated the presence of donor strain antigens in the spleen and lymph nodes of tolerant mice [14], Trentin & Session were the first to report lymphoid cell chimaerism based on cell marker studies [15, 16]. This followed on from earlier studies which utilized the T6 chromosome marker to show that irradiation chimaeras become repopulated and remain donor lymphoid chimaeras [17]. More recently, Kilshaw (personal communication) carried out an autoradiographic assessment of the number of donor cells in CBA mice injected neonatally with (A × CBA) F1 cells and his results of 2–5% agreed closely with those suggested by the *in vivo* sensitisation test [14]. Trentin and Session showed a much higher percentage of donor lymphoid cells in tolerant mice ranging from 17 to 94%, and that the highest number of donor cells (94%) was found in their spleen preparations [15, 16]. Our results ranged from 2 to 100% donor cells. These were confined to the spleens of 9 of our 36 animals tested by chromosomal analysis. There was no AKR "takeover" as seen in our earlier studies with the E.E.A. chimaeras [3, 4]. Although all our mice were highly tolerant as judged by persistence of skin grafts we were unable to confirm chimaerism in all animals, either cytogenetically or by analysis of cell products. This was in spite of analysing more than 1600 mitoses from preparations of blood, thymus, spleen, bone marrow, lymph node and occasionally other tissues.

The incidence of lymphoma in the tolerant AKR was in no way different to that seen in the untreated AKR controls. This was independent of whether or not the tolerant AKR received CBA thymus grafts or lymphoid cells.

Conversely, the effect of induction of tolerance with or without AKR thymus grafting had a marked effect upon the CBA. Lymphomas occurred in the majority of the tolerant CBA but with delayed incidence comparable to the (AKR × CBA) F1 [18]. Unlike the lymphomas in the AKR, which frequently result in enlargement of the thymus, those seen in the tolerant CBA, like the F1, generally presented with generalized lymphadenopathy without an obvious thymoma. The significance of this finding is uncertain but the difference in presentation of the disease could reflect the influence of host factor control. The overall results differ from those of Miller in which he found that no lymphomas developed in low-leukaemic C3H mice made tolerant of AKR tissue by intravenous injection of AK_1 lymphoid cells at birth [19, 20].

The sequence of events leading to tumour development in our tolerant CBA remains unknown. It is known, however, that a graft of an AKR thymus into a relatively resistant tolerant recipient confers tumour susceptibility but that the resulting tumours are sometimes of host origin [19]. This appears to be the case in our tolerant CBA where all the lymphomas possessed the T6 marker of the host. One animal was especially interesting since although the lymphomatous mesenteric lymph node was 100% CBA as judged by cytogenetic analysis, the lymphomatous spleen was 100% AKR. This suggests the possibility of polyclonal lymphoma development in this mouse. This is also in evidence in Miller's studies which showed that lymphocytic neoplasms developed in thymectomised tolerant C3H mice bearing subcutaneous AKR thymus grafts. These tumours were transplantable into either host, donor or both strain recipients [19].

Since our lymphomas were of host origin and unlike Miller's findings were not confined to the AKR thymus grafted tolerant CBA, tumour susceptibility cannot be attributed to the direct influence of the AKR thymus graft. Neither can it be that the AKR spleen cells used to induce tolerance have seeded an AKR clone of cells predestined to AKR lymphoma development. Tumour de-

velopment in these tolerant mice, therefore, appears to be most likely associated with AKR-MuLV infection from the injected AKR spleen cells. There is good evidence for this since levels of p30 were elevated and comparable with normal AKR. This is not surprising since the CBA, like the AKR, are Fv-1^n and H-2^k-genetic loci associated with permissiveness to N-tropic (AKR) MuLV infection [21] and virus associated tumour development [22].

The fact that Miller failed to demonstrate that the neonatal injection of AK_1 cells *per se* produced leukaemia in tolerant thymectomised C3H mice bearing syngeneic thymus grafts [19] or in tolerant C3H mice with intact thymuses [20] is interesting. He concluded that the AKR cells injected were unable to induce leukaemia in their host e.g. by releasing virus, in spite of the fact that the C3H are Fv-1^n and H-2^k are very susceptible to injection of leukaemogenic filtrate. In retrospect, it must however be remembered that subline differences are very important and Miller's C3H/PW subline in 1960 was more refractory than for example the C3H/Gs subline at that time to even his most powerful Passage A leukaemogenic filtrate. It would, therefore, seem reasonable to postulate that genetic influences other than those dependent on the Fv-1^n, H-2^k genes must have played some role in enabling C3H/PW mice to resist leukaemogenesis by any virus present in the tolerizing inoculum.

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