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Failure to Detect Anti-Group-specific Murine Leukemia Virus Activity in Tetraparental AKR-CBA Chimeras

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SUMMARY

Tetraparental AKR-CBA/H-T6 chimeras were primarily derived and investigated to determine whether factors associated with the tumor resistance of the CBA/H-T6 could overcome the innate lymphoma susceptibility of the AKR. Evidence has since shown that, on comparison with the AKR, lymphomas were not only delayed but were also less common in a group of 18 early embryo aggregation-derived AKR-CBA/H-T6 tetraparental chimeras.

Evidence here has shown other clear differences between the AKR and AKR-CBA/H-T6 chimeras. Whereas murine group-specific murine leukemia viral antigens were detected in the sera in both situations, immunoabsorption studies showed that, in the AKR, the antigens exist complexed to the corresponding antibodies. The situation in the chimeras was in complete contrast, since here antigens exist as a "free" form. This in turn has led us to suggest that the advantage in respect to tumor immunity in the AKR-CBA/H-T6 chimeras is due to the tolerance to oncogenic virus being maintained. In this situation and in contrast to the AKR, in the absence of "masking" antibody-viral antigenic complexes, "normal" tumor immunity can be effected. It has to be assumed that tolerance to the oncogenic Gross virus in the AKR-CBA/H-T6 chimeras reflects the influence of the CBA component. How this has possibly been achieved is discussed.

INTRODUCTION

Since the proportion of the 2 parental strain cell populations is known to change in tetraparental mouse chimeras (9, 14), it was conceivable that tumor resistance of the AKR-CBA/H-T6 chimeras (8) might have been due to the loss of the lymphoma-prone AKR cell population. Cytogenetic analysis, however, has since shown that this was not the case. Analysis of phytohemagglutinin-stimulated peripheral blood cultures revealed an overwhelming prevalence of AKR cells (>99%) (16). Predominance was not confined to phytohemagglutinin-stimulated blood cultures, since analysis of direct unstimulated preparations of the majority of tissues in nearly all the chimeras also showed abundant AKR mitoses (>85%) (11). Tumor resistance of the chimeras therefore could not be attributed to the absence of lymphoma-prone AKR cells, but there was 1 possible argument against this assumption. Cytogenetic analysis is generally restricted to cells in division; therefore it was conceivable that the apparent AKR excess might have only reflected an increased mitotic index in the AKR. However, this does not appear to be the case, since AKR cells divide less actively than do CBA cells during culture of an artificial mixture of these cells (16). Analysis of the red cell isozyme glucose phosphate isomerase and serum allotype levels also showed AKR predominance (5). Tumor resistance of the chimeras therefore could not be attributed to either the lack of lymphoma-prone AKR cells (12,18), or their products (5).

Tumor resistance of the chimeras also could not be attributed to the absence of the oncogenic Gross virus. Numerous type-C MuLV[1]-like particles were observed by electron microscopy (17), and titers of Gross (*gs*) antigen comparable with the AKR were recorded (7). One curious fact about the AKR is that, in spite of the early acquisition of oncogenic Gross virus upon the germ line (4), tolerance is short lived. From about the age of 3 months, antiviral antibodies develop and these complex with the corresponding viral antigens (15). Such complexes are best seen by means of immunofluorescence as "lumpy-bumpy" staining of the renal glomeruli (15). In striking contrast to the AKR, renal complex staining the chimeras was minimal (7). Since this could not be explained by the lack of *gs* antigen (7), the absence of corresponding antibody seemed the most probable explanation, and this has been examined here.

MATERIALS AND METHODS

The experimental design was based upon the fact that *gs* antigen is known to be present in the sera of both the AKR (12) and the AKR-CBA/H-T6 chimeras (7). In the AKR the antigen exists in the serum complexed with the corresponding antibody (15). We sought to learn whether this is also true in the chimeras, since if it could be shown that the antigen was free, then this in turn would support our view concerning the absence of *gs* antibody in the AKR-CBA/H-T6 chimeras.

An indirect 2-stage immunofluorescent absorption assay was used for quantitation of *gs* antigen (12). The 1st stage involved primary titration of the specific goat anti-MuLV-*gs*-1 serum (the serum was kindly donated by Dr. R. Gilden, Flow Laboratories, Rockville, Md.) against fixed AKR lymphoma cells (7). After treatment and washing, fluores-

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[1] The abbreviation used is: MuLV, murine leukemia virus.

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cein-labeled heterologous anti-goat serum was applied and the end point was determined according to previously described criteria (12). Following titration of the anti-MuLV-*gs* serum, it was used at 2 dilutions greater than the previously determined end point in the immunofluorescent absorption technique (12). In practice, doubling dilutions of the mouse sera were incubated (4° overnight) with an equivalent volume of the diluted antiserum. The absorbed antiserum was then used in the indirect immunofluorescent test and its end point was again determined. The antigen titer was expressed as the reciprocal of the corresponding dilution of the antigen that was shown to absorb out activity. For example, a titer of 1 refers to the absorption by an amount of MuLV-*gs* antigen contained in neat serum, whereas an antigen titer of 4 refers to successful absorption by 1:4 dilution of antigen and so on.

Indirect absorption was used in an attempt to demonstrate the lack of anti-MuLV-*gs* activity in the sera. For convenience, anti-mouse immunoglobulin was coupled to Sepharose and this was used for immunoabsorption. Two different Sepharose suspensions were used in each case. The 1st was untreated and the 2nd was coupled to a crude globulin fraction [50% saturated $(NH_4)_2SO_4$] of rabbit anti-mouse immunoglobulin (IgG and IgM). Coupling was achieved by means of cyanogen bromide (18) and was confirmed after washing by means of immunofluorescence using fluorescein-labeled anti-rabbit serum. Fluorescein-labeled anti-mouse serum was used to exclude nonspecific mouse protein binding.

The same volume and approximately the same concentration of each Sepharose suspension was used in each instance for treatment of an equivalent volume of serum. Following incubation (4° overnight), the serum samples were centrifuged and the supernatants were assayed for *gs* activity as described above. Any fall in *gs* titer of the supernatant following treatment with anti-immunoglobulin-coupled Sepharose (compared with the uncoupled Sepharose) was held to reflect the loss of *gs* activity following anticipated reduction in serum antibody (immunoglobulin) due to successful immunoabsorption.

The Sepharose deposits were subsequently resuspended, washed 3 times in phosphate-buffered saline (Dulbecco A; pH 7.2), and examined by direct fluorescence after treatment with fluorescein-labeled anti-mouse and anti-rabbit sera. "Blocking" controls were included, and all samples were examined upon a double-blind basis.

RESULTS

It can be seen from Table 1 that the 2 AKR control serum pools, both prepared from aged AKR with lymphomas, had relatively high titers of *gs* antigen, while no activity was demonstrated in the single pooled CBA serum sample.

gs antigen levels in the chimera sera were similarly determined and, as can be seen from Table 2, reciprocal titers of 1 to 16 were obtained. These results were obtained upon samples pooled from individual blood samples taken at varying intervals between 20 and 40 to 90 weeks of age, depending upon the actual chimera. Although there was no obvious association between the *gs* titer and the development of lymphoma, the titers appeared to be greatest in the predominantly albino (AKR) mice. Although titers were on occasion higher than the AKR controls, it must be remembered that the chimeras were generally much older than were the AKR, and the 2 groups are therefore not strictly comparable.

Dilution errors probably occurred in spite of the use of unit volume, attempts to maintain a constant concentration of the Sepharose suspension, and the necessity of being restricted to the use of only minute volumes (20 μl of both serum and Sepharose), which limits interpretation of the results. In spite of this, certain conclusions are apparent from the results summarized in Table 3. Although a reciprocal titer of 4 was originally obtained in the *gs* assay of AKR serum pool B, no antigen was detected after treatment with uncoupled Sepharose. This apparent loss of activity was not due to nonspecific binding, since immunofluorescence failed to demonstrate any mouse serum coating. Dilution with the buffer necessarily used to suspend the Sepharose suspensions is the most probable explanation. This also appears to be the case with the chimera samples. Whereas all of the chimeras were positive on initial testing (Table 2), only 7 of the 14 were positive (13 of 27 assays) following treatment with uncoupled Sepharose (Table 3).

Table 1

gs antigen titers in the serum of 2 groups of AKR mice and a group of CBA mice

Source of sera	Age (wk)	Serum dilution								Titer (reciprocal)
		Neat	1:2	1:3	1:4	1:5	1:6	1:7	1:8	
AKR-GpA	36	–	–	NT[a]	–	NT	–	–	+	7
AKR-GpB	44	–	–	–	–	+	+	+	+	4
CBA	40–46	+	+	+	+	+	+	+	+	0

[a] NT, not tested.

Table 2

Titer of gs antigen in sera of AKR-CBA/H-T6 chimeras

Association of albino (AKR) coat color composition with the serum gs antigen titers in a group of AKR-CBA/H-T6 chimeras.

AKR-CBA/H-T6 chimera	% of AKR in coat	Lymphoma	*gs* titer (reciprocal)
1	95	+	16
2	90	–	8
3	90	+	4
4	90	–	16
5	80	–	16
6	80	+	8
7	70	–	4
8	70	–	4
9	60	+	16
10	60	+	4
11	60	–	2
12	50	+	2
13	45	–	8
14	40	–	4
15	35	–	4
16	25	–	2
17	5	–	4
18	0	–	2

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gs antigen could not be demonstrated in either of the AKR control serum pools after treatment with the anti-mouse Ig-coupled Sepharose (Table 3). This was of no significance in the case of the AKR B serum pool, since, as mentioned above, no activity was demonstrated after treatment (dilution) with uncoupled Sepharose. Results with AKR A serum pool were more interesting. Although *gs* antigen activity was detected after treatment with uncoupled Sepharose (positive in all 3 tests), no activity could be demonstrated in the supernatant after incubation with coupled Sepharose (negative in all 5 tests). This suggested the secondary binding of *gs* antigen to the Sepharose due to a primary rabbit anti-mouse to mouse immunoglobulin reaction. This was subsequently confirmed, since incubation of these particles with an aliquot of MuLV-*gs* serum was shown to reduce the titer in the indirect immunofluorescent assay from 1:512 to 1:126. Reduction of anti-MuLV-*gs* activity thus confirmed the presence of *gs* antigen on the anti-Ig-coated Sepharose, and this in turn suggested that, in this situation, the *gs* antigen was complexed to mouse immunoglobulin.

The situation was completely reversed in the chimeras. Here there was no loss of *gs* activity following incubation with the anti-Ig-coupled Sepharose (Table 3). Surprisingly, positive results were more common than after treatment with uncoupled Sepharose. Unavoidable and varying dilutions of the 2 Sepharose preparations appear to be the most probable explanations. The fact that *gs* antigenicity was unaltered in the chimeras following treatment with anti-Ig-coupled Sepharose suggests that *gs* antigen is free in this situation. As can be seen from Table 3, immunofluorescence clearly demonstrated successful binding with mouse serum. Immunoelectrophoresis confirmed this, since markedly reduced levels of γ-globulin were noted in the serum samples following treatment with the anti-Ig-coupled Sepharose (J. Holliday and R. D. Barnes, unpublished data). The fact that the coated Sepharose particles used for absorption of the chimera sera samples failed to neutralize anti-MuLV-*gs* serum is further evidence to support the lack of binding of the *gs* antigen. All evidence therefore favors the existence of free *gs* antigen and, in turn, the absence of anti-*gs* antibody activity in the chimera.

DISCUSSION

Various antigens have been associated with the type-C murine viruses. These are in general either associated with the virion or, alternatively, the cell surface. Unfortunately, we were only able to investigate for the presence of antibody against the *gs*-1 antigen located on the envelope of the virion. In spite of this limitation, the findings appear significant.

Table 3

gs antigen in AKR-CBA chimeras and AKR sera after treatment with immunoabsorbed anti-mouse Ig and subsequent specificity of the absorbent when tested in direct fluorescence

Results of *gs* antigenic activity in AKR and AKR-CBA/H-T6 chimeras both before and after immunoabsorption, designed to render the sera relatively agammaglobulinemic.

Mouse	Presence of lymphoma	Treated with noncoupled Sepharose			Treated with anti-Ig-coated Sepharose		
		gs serum activity[a]	Fluorescent staining of Sepharose[b]		*gs* serum activity[a]	Fluorescent staining of Sepharose[b]	
			Anti-rabbit	Anti-mouse		Anti-rabbit	Anti-mouse
AKR							
A pool	+	+, +, +	–	–	–, –, –, –, –	++	+
B pool	+	–, –	–	–	–, –, –	++	+
AKR-CBA							
1[c] (95)	+	+, –	–	–	+, +	++	+
3 (90)	+	+, +	–	–	+, –	++	+
4 (90)	–	–, –	–	–	+, –	++	+
5 (80)	–	+, +, +, +	–	–	+, +, –	++	+
6 (75)	+	–, –	–	–	+	++	+
7 (70)	–	+, –	–	–	+	++	+
8 (70)	–	–	–	–	+	++	+
9 (60)	+	+, +	–	–	+	++	+
10 (60)	+	+,+,–	–	–	+, –	++	+
12 (50)	+	–, –	–	–	+, –	++	+
13 (45)	+	–	–	–	–	++	+
16 (25)	–	+, –	–	–	+, –, –	++	+
17 (5)	–	–	–	–	–	++	+
18 (0)	–	–	–	–	–	++	+

[a] Individual results.

[b] Particle fluorescence arbitrarily assessed – → + → ++.

[c] Chimera number refers to earlier described findings (4, 7); numbers in parentheses, approximate percentages of AKR albino coat color composition.

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Although *gs* antigen was found in all of the AKR-CBA/H-T6 chimeras, the highest titers in the sera were detected in the predominantly albino (AKR) chimeras. A similar correlation was noted between the numbers of type-C MuLV-like particles and coat color composition (17). Both results are surprising in the context of the general and overwhelming predominance of AKR mitoses seen in all of the chimeras (11, 16). Under these circumstances one might have anticipated seeing an AKR "pattern" with numerous type-C particles and large amounts of *gs* antigen. Since the viral content appears to be independent of the AKR mitotic activity and the overall AKR cellular composition of each chimera, from these results it would appear that the extent of viral replication is controlled by a process established in early embryonic life, and furthermore this is related to coat color composition. Evidence therefore implicates a factor in the CBA (? coat) that influences Gross viral replication. More important is the fact that this can influence viral replication in AKR cells. This is a particularly startling observation, since, like the AKR, the CBA is also $Fv\text{-}1^n$ at the locus that determined permissiveness of replication of activated N-tropic viruses (13). If our CBA had been $Fv\text{-}1^b$, and since resistance is dominant (13), then one might have anticipated overall intercellular restriction of $Fv\text{-}1^n$ viral replication in the AKR-CBA/H-T6 chimeras. However, our CBA are $Fv\text{-}1^n$ positive. Perhaps more important is the observation that the extent of viral restriction appears to be directly related to CBA coat color composition. This suggests the presence of an additional factor associated with CBA coat color that influences viral replication in AKR cells. The fact that this factor can manifest its effect on an overwhelming preponderance of AKR cells in widely distributed sites suggests that this factor is probably soluble (11). As mentioned, 1 possible site of its production might be the stromal elements of the thymus, which, like skin, are also ectodermally derived.

The relative tumor resistance of the chimera has been attributed to the influence of the CBA component (2, 3). The CBA component in the chimeras is also necessarily involved in maintaining tolerance to the Gross virus. These 2 factors might be related and might also be associated with the apparent association of CBA coat color and viral replication. There appears to be a vital clue to favor this possibility. Elsewhere, (10) we have discussed evidence that has raised the possibility that AKR T-cells in the chimeras have been processed with CBA θ, the thymus-associated antigen, a process that conceivably may be directly involved in maintaining tolerance to the Gross virus; this in turn is relevant to the tumor resistance of the chimeras. Although the primary source of θ antigen has yet to be determined, a distinct possibility is the stromal elements of the thymus. It is an attractive hypothesis to consider that thymic stromal contribution of the chimeras has not only influenced the extent of viral replication but has also played a part in maintaining tolerance to the oncogenic virus. This, like the role of θ itself, has yet to be substantiated. In the context of tolerance to the virus being maintained, and in the subsequent absence of masking of tumor-specific sites by antibody-viral antigen complexes, this would enable normal tumor immunity to be effected. This hypothesis would have been supported if masking anti-*gs* antibody had been detected in those few chimeras that developed lymphomas. Although this was not the case, it must be remembered that the serum samples used here were obtained over a long period of time and generally at a stage when no lymphoma was present. It is conceivable that antiviral blocking antibodies may have developed at a later stage in the small group of chimeras that developed lymphomas. This, however, cannot be proven at this stage.

In the context of the possible CBA θ processing of AKR cells, the maintenance of tolerance to the Gross virus, and the tumor resistance of the chimeras, it was interesting to note a recent report by Acton *et al.* (1). These workers noted that 2 AKR sublines, namely AKR/RuA and AKR/CuM, possessed θ C3H rather than θ AKR. These sublines were also exceptional in the respect of being relatively lymphoma resistant. This might be considered secondary evidence to support the possible association of lymphoma resistance with the θ status. We are continuing to test the hypothesis that the relative tumor resistance demonstrated in the AKR-CBA/H-T6 chimeras (6) is associated with θ C3H processing of AKR cells and that this serves to maintain tolerance to the Gross virus, and, in the absence of masking anti-viral antibody, normal tumor immunity is effected.

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