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Antibody responses in resistant and susceptible inbred mice infected with *Trypanosoma congolense*

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SUMMARY

Antibody responses were evaluated in inbred mice previously shown to be susceptible (A/J) or resistant (C57BL/6J and B_6AF_1 hybrid) to infections with relatively avirulent *Trypanosoma congolense*. Titres and the isotype distribution antibodies specific for the trypanosome variant surface glycoprotein (VSG) were determined by indirect immunofluorescence in sera of mice after primary infections with *Trypanosoma congolense* and after challenge infections with the same variant following drug cure. The results of these investigations showed that, during active infection, resistant mice made relatively strong VSG-specific IgM antibodies. This isotype also predominated in challenge infections with the homologous variant following drug cure. In contrast, A/J mice made little or no VSG-specific antibody on first exposure to *T. congolense*. However, these animals were able to produce substantial amounts of protective VSG-specific IgG antibody after multiple-challenge infections with the homologous variant. Substantial titres of VSG-specific antibodies in resistant mice did not influence the numbers of trypanosomes in the first parasitaemic peak as initial parastiaemias were similar in both C57BL/6J and A/J mice. However, C57BL/6J mice cleared parasites in this peak, whereas A/J mice did not. Mice of both strains immunized by infection cure were equally effective in clearing parasites when challenged with homologous trypanosomes. It is clear from the results of this study that antibody is not the sole factor contributing to murine resistance to African trypanosomes.

INTRODUCTION

Natural and experimental infections with African trypanosomes are characterized by significant elevations in serum immunoglobulins thought to occur as a result of the polyclonal activation of B lymphocytes (Greenwood, 1974). Although much of this antibody is considered not to be specific for trypanosomes, there is a considerable amount of evidence to suggest that antibodies directed to the trypanosome variant surface glycoprotein (VSG) are involved in the clearance of trypanosomes in successive parasitaemic waves (Mansfield, 1981; Campbell, Esser & Weinbaum, 1977). However, their precise role in this process and in resistance to African trypanosomiasis has not been elucidated, and there is increasing evidence to indicate that antibody responses are not the only factors which determine susceptibility differences in experimental African trypanosomas.

In an earlier study (Mitchell & Pearson, 1983), we showed that susceptible (A/J) and resistant (C57BL/6J) mice responded differentially in terms of immunoglobulin isotype and repertoire when injected with trypanosome antigens. Resistant mice made qualitatively and quantitatively superior specific IgM responses, particularly to the trypanosome major variant specific glycoprotein. These studies were conducted with trypanosome lysates in order to measure the innate ability of susceptible and resistant mice to produce specific antibodies in a situation in which the parasite itself did not influence the immune response by its metabolic or other processes. In order to determine if resistant and susceptible mice would respond in a similar fashion to active infections with African trypanosomes and to establish if there were temporal differences in the onset of the humoral response, we measured anti-VSG antibodies in resistant (C57BL/6J and B_6AF_1 hybrid) and susceptible (A/J) mice during infections with *Trypanosoma congolense* ILRAD 588. This trypanosome stabilate was used because it causes subacute infections in mice which resemble those in humans and cattle. Previous investigations (Morrison *et al.*, 1978) have shown that C57BL/6J mice and A/J mice exemplify the extremes of resistance and susceptibility to infections with this stabilate.

We also evaluated the host-protective qualities of the anti-VSG antibody response in both resistant (C57BL/6J) and susceptible (A/J) mice by immunizing by infection and drug cure, and challenging with the homologous parasite. In all

Abbreviations: DEAE, diethylaminoethyl; FCS, fetal calf serum; i.p., intraperitoneal; PSG, phosphate-buffered saline-glucose; VSG, variant surface glycoprotein.

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experiments, the isotype distribution of the specific antibody response was determined. The results of these investigations showed that resistant mice responded to trypanosome antigens during active infection and after immunization by making VSG-specific antibodies of the IgM isotype. In contrast, susceptible mice made very little VSG-specific antibody during primary infections with *T. congolense*. However, these animals made substantial amounts of VSG-specific IgG antibody during challenge infections with homologous trypanosomes after drug cure.

MATERIALS AND METHODS

Animals

A/J and C57BL/6J mice were obtained from the Jackson Laboratory, Bar Harbor, ME. B_6AF_1(A/J × C57BL/6J) hybrid mice and Long Evans rats were bred from stock purchased from the Jackson Laboratory and Charles River (Canada) Inc., St Constant, Quebec, respectively. Male and female mice aged 2–5 months at the onset of experiments were used. Mice were age- and sex-matched in all experiments. Male or female rats aged 2 months were used to grow trypanosomes for stabilates and for immunofluorescence assays.

Trypanosomes

Trypanosoma congolense ILRAD 588, a triply cloned stabilate originally derived from an infected bovid (Morrison *et al.*, 1978), was obtained from Dr W. I. Morrison of the International Laboratory for Research on Animal Diseases, Nairobi, Kenya. Parasites for cryostabilates or for immunofluorescence assays were obtained by intraperitoneal (i.p.) injection of 1×10^7–1×10^8 trypanosomes into cyclophosphamide-immunosuppressed (Smith, Levine & Mansfield, 1982) Long Evans rats. At peak parasitaemia (8–10 days after injection), trypanosomes were separated from infected rat blood by DEAE-cellulose chromatography and washed once with phosphate-buffered saline-glucose (PSG) (Lanham & Godfrey, 1970). Trypanosomes were used directly in immunofluorescence assays or cryostabilates were prepared by resuspending trypanosomes in PSG containing 10% fetal calf serum (FCS) and 10% glycerol and freezing in liquid nitrogen. Cryostabilates were thawed just before use in a 37° water-bath, and trypanosomes were adjusted to the appropriate concentrations in sterile PSG.

Parasitaemia determinations

Tail blood was monitored for trypanosomes from the third or fourth day following i.p. injection of *T. congolense* by examining wet films of whole blood. Parasite numbers were estimated by the 'matching method' (Herbert & Lumsden, 1976) during the early stages of infection. After Day 5 (post-injection), parasite numbers were determined by haemocytometer after diluting the blood in PSG containing Aerolysin toxin as previously described (Pearson *et al.*, 1982).

Immunization by infection and drug cure

Five to six mice of each strain were infected by i.p. injection with living *T. congolense* parasites. Approximately 7–8 days later, the mice were given the trypanocidal drug, Berenil (diminazene aceturate, Calbiochem-Behring, La Jolla, CA, 0·5 mg i.p. in 0·1 ml of sterile saline). Tail blood was monitored for parasites over the following 48 hr to ascertain cure. Ten to 15 days after Berenil cure, mice were bled via the caudal vein. Sera were prepared and stored at −20° until tested (primary immune sera). Mice were challenged with the homologous variant and tail-bled at 4 days and 10 days following challenge. Sera were prepared and stored at −20° (secondary immune sera).

Antisera

Affinity-purified goat anti-murine IgM heavy chain (anti-μ) and goat anti-murine IgG heavy chain (anti-γ) sera conjugated with fluorescein were purchased from Kirkegaard-Perry Laboratories, Gaithersburg, MD. Fluorescein-conjugated goat anti-

Table 1. Anti-VSG antibodies in inbred mice during primary infections with *T. congolense*

No. trypanosomes injected	Day of infection	Mouse strain	Reciprocal antibody titre		
			IgM + IgG	IgM	IgG
Low (5×10^3)	4	A/J	< 50	ND*	ND
		C57BL/6J	50	ND	ND
	9	A/J	< 50	ND	ND
		C57BL/6J	1250–6250	ND	ND
Intermediate (7×10^4)	3	A/J	250–1250	ND	ND
		C57BL/6J	6250	ND	ND
High ($2{\cdot}5 \times 10^5$)	4	A/J	0	0	0
		C57BL/6J	< 50	0	0
	10	A/J	50	50	50
		C57BL/6J	1250–6250	1250	50

IgM and IgG titres were determined using FITC-conjugated anti-μ and anti-γ chain sera, respectively. Antibodies of both isotypes (IgM + IgG) were detected with a FITC-conjugated antiserum directed to both murine immunoglobulin classes.

* ND, not determined.

murine IgM + IgG antiserum was obtained from Gibco, Burlington, Ontario. Optimal working dilutions were determined and specificity was ascertained by testing these antibodies in enyzme-linked immunosorbent assays (Voller, Bidwell & Bartlett, 1976) using affinity-purified murine myeloma proteins as target antigens.

Immunofluorescence assays with living trypanosomes

Surface-directed (anti-VSG) antibodies in the sera of *T. congolense*-infected mice were titrated in immunofluorescence assays using living homologous DEAE-cellulose-purified *T. congolense* as previously described (Pearson *et al.*, 1981). Slides were prepared immediately before examination. Surface-bound fluorescent antibody was detected using a Zeiss standard microscope fitted with an epifluorescence attachment with an ultraviolet illuminator and 63 × Neofluar oil immersion objective. The end-point of the titration was taken as the serum dilution showing perceptible fluorescence above background levels found in negative controls. Results are expressed as the reciprocal of this serum dilution.

RESULTS

Specific antibody responses to trypanosome surface antigens in *T. congolense*-infected mice

Primary infections. VSG-specific antibody titres were measured in A/J, C57BL/6J and B_6AF_1 mice at early intervals during primary infections with *T. congolense* ILRAD 588 in several experiments. In some assays, sera from individual animals were tested separately. In others, sera were pooled from several animals of each strain at each time and tested as a single sample.

Table 1 shows the antibody titration data for the sera from female mice infected with varying doses of *T. congolense*. Sera collected from five to six mice of each strain at each time-point were pooled and tested. At all doses of trypanosomes, anti-VSG antibody titres were higher in the sera of C57BL/6J mice than in A/J mice at both 3–4 and 9–10 days following i.p. injection of trypanosomes. The data at the bottom of Table 1 show that most of the anti-VSG antibodies in C57BL/6J mice were IgM, whereas A/J mice made approximately equal amounts of IgG and IgM.

In order to determine the degree of variation within and between mouse strains, sera collected 3 days after i.p. injection of 7×10^4 *T. congolense* organisms were retested individually in five mice of each strain. Data from these experiments are shown in Table 2. Three days after trypanosome injection, IgM titres were 570 ± 281 and 4010 ± 1389 ($\bar{x} \pm$ SE) for A/J and C57BL/6J mice, respectively. IgG titres did not differ appreciably between mouse strains at this time, being 850 ± 246 and 760 ± 301 ($\bar{x} \pm$ SE) for A/J and C57BL/6J mice, respectively.

In another experiment, the sera of male A/J, C57BL/6J and B_6AF_1 mice that had been infected with 5×10^3 trypanosomes were tested individually for titres of IgM anti-VSG antibodies. These sera were also tested as pools for the determination of levels of VSG-specific IgM and IgG. The data from these

Table 2. Primary anti-VSG antibody responses in individual mice infected* with *T. congolense*: Day 3 of infection

Mouse strain	Observation number	Reciprocal antibody titre		
		IgM + IgG	IgM	IgG
A/J	1	ND†	1250	1250
	2	ND	1250	1250
	3	ND	250	1250
	4	ND	50	250
	5	ND	50	250
	Pooled sera	250–1250	ND	ND
C57BL/6J	1	ND	6250	1250
	5	ND	250–1250	50
	Pooled sera	6250	ND	ND

* Mice were infected with 7×10^4 trypanosomes.
† ND, not determined.

Table 3. Anti-VSG antibodies in mice infected* with *T. congolense*

Day of infection	Mouse strain	Observation number	Reciprocal antibody titre	
			IgM	IgG
5	A/J	1	< 50	ND†
		2	< 50	ND
		3	< 50	ND
		Pooled serum	< 50	< 50
	C57BL/6J	1	250	ND
		2	250–1250	ND
		3	250–1250	ND
		Pooled serum	250–1250	< 50
	B_6AF_1	1	< 50	ND
		2	< 50	ND
		3	250	ND
		Pooled serum	ND	ND
8	A/J	1	250–1250	ND
		2	50–250	ND
		3	50	ND
		Pooled serum	250	< 50
	C57BL/6J	1	6250	ND
		2	1250–6250	ND
		3	250–1250	ND
		Pooled serum	6250	< 50
	B_6AF_1	1	250–1250	ND
		2	6250	ND
		3	1250–6250	ND
		Pooled serum	ND	ND

* Mice were infected with 5×10^3 trypanosomes.
† ND, not determined.

experiments are summarized Table 3. Very little or no parasite-specific IgG antibody was made by either A/J or C57BL/6J mice. IgG titres were not determined in B_6AF_1 mice. As was observed with higher doses of *T. congolense*, IgM antibodies appeared earlier and were of higher titre in the sera of C57BL/6J mice. One B_6AF_1 mouse had moderate titres of IgM anti-VSG antibody by Day 5 of infection. By Day 8 of infection, all hybrid mice made IgM anti-VSG antibodies.

Anti-VSG antibody responses after challenge infections with T. congolense *in mice immunized by infection cure.* VSG-specific antibody responses were measured during challenge infections with homologous *T. congolense* in A/J and C57BL/6J mice that had been immunized by one (primary immune) or two (secondary immune) rounds of infection followed by Berenil cure. Groups of five to six female mice (aged 2–3 months) of each strain were used. Normal age- and sex-matched mice that had been injected with Berenil at the same times as the infected mice served as unimmunized controls. After mice had been immunized, all animals were challenged by i.p. injection of homologous parasites. Anti-VSG antibody titres were determined by immunofluorescence on living homologous trypanosomes. Several experiments were conducted.

In the experiment shown in Table 4, parasite-specific antibody titres were followed in C57BL/6J and A/J mice during priming and two successive challenge infections with 2×10^5 *T. congolense* organisms. Sera were tested as pools from five mice of each strain. Anti-VSG antibody titres after the priming infection and during the first challenge infection were higher in the sera of C57BL/6J mice than in A/J mouse serum. During the second challenge, although anti-VSG titres were initially higher (Day 4) in the serum of C57BL/6J mice, by Day 10 antibody titres were the same in both strains. As was observed in the other experiments, the bulk of the parasite-specific antibody response of C57BL/6J mice to challenge infections was IgM with very little IgG being made. In contrast, although A/J mice made approximately equivalent amounts of anti-VSG antibodies of both isotypes during the first challenge infection, they switched to the production of IgG during the second challenge infection.

Table 4. Anti-VSG antibodies in mice after repeated challenge with *T. congolense*

Treatment	Reciprocal antibody titre		
	IgM + IgG	IgM	IgG
Prechallenge antibody titres*			
A/J	> 50	ND§	ND
C57BL/6J	> 6250	ND	ND
Antibody titres after first challenge†			
Day 4			
A/J	250	1250–6250	1250
C57BL/6J	> 6250	6250	250
Day 9			
A/J	250	1250	1250
C57BL/6J	250	6250	250
Antibody titres after second challenge‡			
Day 4			
A/J	1250	1250	1250
C57BL/6J	1250–6250	6250	250
Day 10			
A/J	6250	1250	6250
C57BL/6J	6250	6250	250

* Anti-VSG titres 10 days after Berenil cure of the infection resulting from the first exposure to *T. congolense* ILRAD 588.

† Anti-VSG titres during first challenge infection (16 days after Berenil cure) with homologous trypanosomes. Animals were given Berenil on Day 25 of this infection.

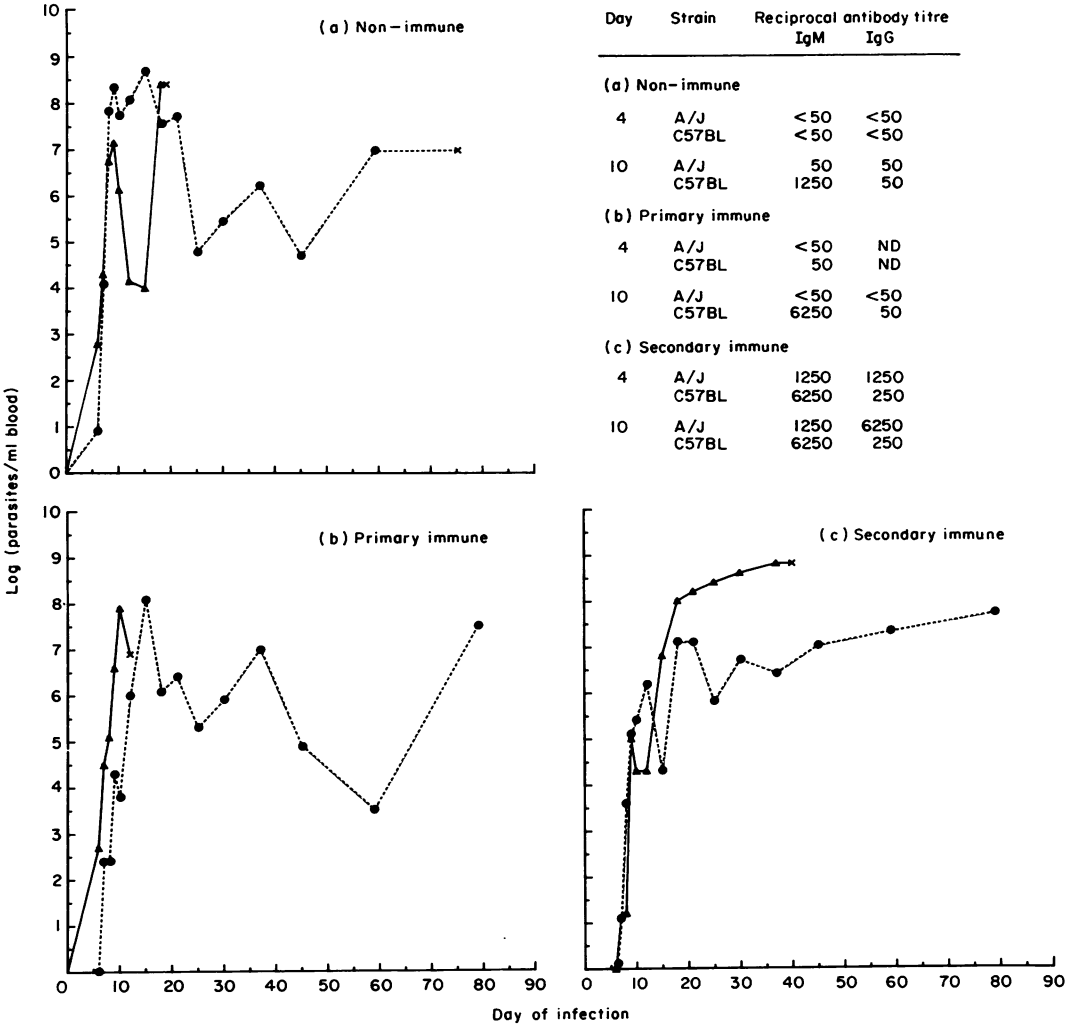
‡ Animals were challenged 1 month after the last Berenil injection with homologous trypanosomes. Anti-VSG antibody titres were measured at the indicated days of this challenge infection.

§ ND, not determined.

Parasitaemia profiles and survival of immunized and unimmunized mice during infections with T. congolense. Parasitaemias were followed and survival determined in groups of A/J and C57BL/6J mice immunized by infection cure. The parasitaemia profiles of primary immune, secondary immune and unimmunized control mice are shown in Fig. 1. Anti-VSG antibody titres on Days 4 and 10 of the last challenge infection are also displayed in Fig. 1. Survival data for these animals are displayed in Fig. 2. The relatively higher antibody titres in C57BL mice did not appear to influence the height of the first peak in the unimmunized group (Fig. 1a), but correlated with a slight reduction in the height of the first peak in the primary immune group (Fig. 1b). In the secondary immune mice (Fig. 1c), the height of the first parasitaemic peak was reduced considerably (in both strains of mice) relative to that observed in the unimmunized and primary immune animals. Secondary immune A/J mice, which made predominantly IgG anti-VSG antibodies, cleared parasites from the first peak as well as, or better than, C57BL mice. However, secondary immune A/J mice were not able to control parasite numbers in the second peak.

A single priming dose of 5×10^3 trypanosomes (Fig. 2b) did not influence survival times in A/J mice: all challenged animals died at approximately the same time as unimmunized control mice (Fig. 2a) when challenged with homologous *T. congolense*. Mean survival times were 10 days and 6·5 days for unimmunized and primary immune A/J mice, respectively. This correlated with the failure of A/J mice to make good titres of anti-VSG antibodies of either isotype. However, secondary immune A/J mice survived considerably longer as a group (mean survival time 20 days), although by Day 20 of infection only two out of the five mice infected remained alive (Fig. 2c). However, these survivors sustained high parasitaemias until their death at Day 40 of infection. In contrast, C57BL mice in all three groups experienced the usual fluctuating parasitaemic waves (Fig. 1c). Prior exposure to trypanosomes reduced the number of early deaths in C57BL/6J mice but did not significantly lengthen survival times of the longest lived animals (Fig. 2c).

In a second experiment in which mice were primed with a higher dose ($2{\cdot}5 \times 10^5$) of trypanosomes and challenged with the same number of homologous parasites, all animals of both strains were solidly immune and did not develop parasitaemias after challenge (data not shown). Unimmunized A/J control mice developed high parasitaemias and were all dead by Day 10 of infection, whereas all unimmunized C57BL/6J control animals experienced fluctuating parasitaemic peaks and survived as previously observed. Control of the first peak correlated with



Day	Strain	Reciprocal antibody titre IgM	IgG
(a) Non-immune			
4	A/J	<50	<50
	C57BL	<50	<50
10	A/J	50	50
	C57BL	1250	50
(b) Primary immune			
4	A/J	<50	ND
	C57BL	50	ND
10	A/J	<50	<50
	C57BL	6250	50
(c) Secondary immune			
4	A/J	1250	1250
	C57BL	6250	250
10	A/J	1250	6250
	C57BL	6250	250

Figure 1. Parasitaemia profiles of non-immune mice, and mice immunized by infection and drug cure in challenge infections with *T. congolense* ILRAD 588. A/J (▲) and C57BL/6J (●) mice (four to six animals of each strain) were immunized by one (primary immune) or two (secondary immune) rounds of infection with *T. congolense* followed by Berenil cure. Normal mice received Berenil at the same times as the infected mice. All mice were challenged with $2{\cdot}5 \times 10^5$ homologous trypanosomes. Each point represents the mean of parasitaemias of the surviving mice in each group. Anti-VSG antibody titres measured in sera pooled from each group of mice on Days 4 and 10 of the challenge infection are also shown. (×) indicates the death of the last animal in each group.

moderately high titre of anti-VSG antibodies of both classes in primary immune A/J and C57BL/6J mice.

DISCUSSION

This investigation has shown that, when actively infected with *T. congolense*, resistant (C57BL/6J and B_6AF_1) mice made substantial titres of VSG-specific IgM antibodies during primary infections and in challenge infections with homologous trypanosomes following cure with the trypanocidal drug Berenil. In these mouse strains, very little IgG anti-VSG antibody was made even after repeated exposure to the same trypanosme variant. With A/J mice actively infected or challenged with live *T. congolense*, the opposite was true. Thus, what we observed (Mitchell & Pearson, 1983) with antibody responses in mice of these strains immunized with soluble antigens of a clone of *T. gambiense* (which did not show differential virulence for different mouse strains) was also seen in active infections with a less virulent clone of *T. congolense* (which does show differential virulence for different strains of inbred mice). This suggests that the isotype of the anti-VSG response is affected by the genetic constitution of the host, in addition to being influenced by parasite virulence as shown by Sacks *et al.* (1980).

Although several studies have shown that parasite-specific IgM is elevated in resistant mice infected with other African trypanosome species (Levine & Mansfield, 1982; Clarkson, 1976) and other *T. congolense* clones (Whitelaw *et al.*, 1980, 1983; MacAskill, 1983), studies involving *T. vivax* infections in mice (DeGee, Shad & Doyle, 1982) showed that superior VSG-specific antibody responses were not correlated with resistance, although good anti-VSG responses were observed in surviving mice from either resistant or susceptible strains. Thus, the results of our study support the former observations, i.e. that VSG-specific IgM antibodies are elevated in resistant mice.

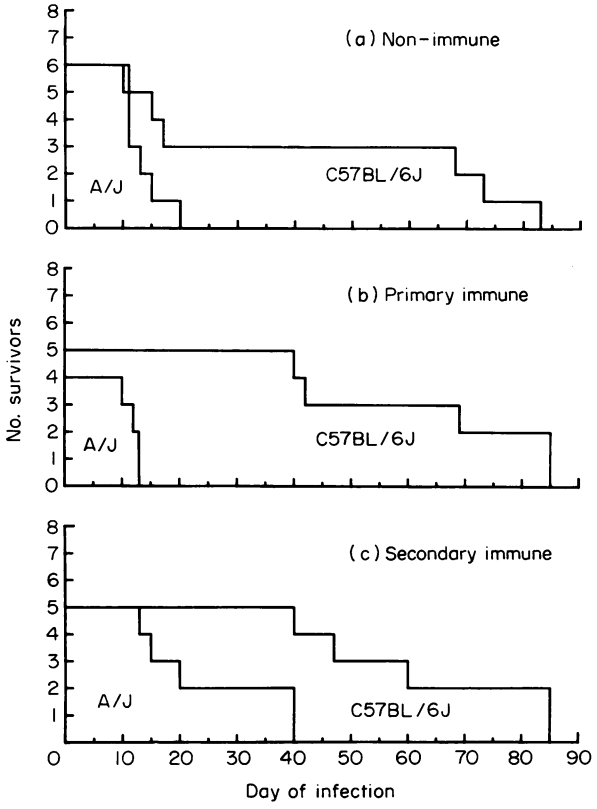


Figure 2. Survival of non-immune mice and mice immunized by infection and drug cure in challenge with *T. congolense*. Details of immunization and challenge infections are given in the legend to Fig. 1.

However, the capacity of resistant mice to make high-titred VSG-specific IgM antibodies did not relate to the height of the first parasitaemic peak on first exposure to *T. congolense* ILRAD 588, as this peak was often higher in C57BL/6J mice than in A/J mice which made considerably lesser amounts of antibody. Thus, high titres of VSG-specific antibodies were not associated with the ability to limit the numbers of parasites establishing themselves in the circulation unless the antibody was performed as in the case of mice (both strains) immunized by infection cure. However, high titres of IgM anti-VSG antibodies did correlate with the ability of resistant mice to clear parasites from the blood and to survive for a longer period of time during primary infections with *T. congolense* ILRAD 588.

In both A/J and C57BL/6J mice immunized by infection cure, high titres of anti-VSG antibodies correlated with a reduction in the height of the first parasitaemic peak upon challenge with live homologous trypanosomes. However, VSG-specific IgG antibody responses were as good as (or better than) VSG-specific IgM in controlling parasite numbers in the first peak, as A/J mice (which made predominantly IgG responses) cleared homologous parasites more rapidly than C57BL/6J mice (which made predominantly IgM anti-VSG antibodies). This could possibly be explained by the observation that IgG antibodies are usually of higher affinity than IgM antibodies.

Thus, the ability to maintain VSG-specific IgM antibody responses appears to be a significant factor in the resistance of C57BL/6J and B_6AF_1 mice to infections with *T. congolense* ILRAD 588. However, genetic studies (Morrison *et al.*, 1978) have shown that resistance to this trypanosome clone is under the control of more than one gene. Therefore, it must be considered that antibodies are only partially responsible for the susceptibility differences observed between mouse strains and may be a reflection of genetic differences in immunoregulatory circuits which also influence immune depression. Sacks & Askonas (1980) have shown that VSG-specific IgG responses are preferentially suppressed in *T. brucei*-infected mice. The tendency of *T. congolense* ILRAD 588-infected A/J mice to produce VSG-specific IgG responses may result in the rapid loss of the ability to produce sufficient antibody. Therefore, genetic differences in resistance to African trypanosomes may reflect only the ability of the animal to continue producing VSG-specific antibodies of either isotype. The real genetic difference (which might account for the predominance of IgM antibodies in resistant mice) may actually lie in immunoregulatory circuits influencing the IgM to IgG switch.

Other humoral responses that influence the differentiation state of bloodstream trypanosomes may also contribute to host resistance by enabling an effective anti-trypanosome antibody response to occur as suggested by Black *et al.* (1983).

Finally, the mechanisms, such as mononuclear phagocyte activation or the ability to accelerate bone marrow haematopoiesis in times of physiological stress, may not only contribute to parasite elimination, but may also be prime determinants of the differences observed between resistant and susceptible mice in the VSG-specific antibody response.

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