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TRYPANOSOMA CRUZI: SERUM ANTIBODY REACTIVITY TO THE PARASITE ANTIGENS IN SUSCEPTIBLE AND RESISTANT MICE

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The specific antibody responses were compared among susceptible (A/Sn), moderately susceptible (Balb/c) and resistant (C57 BL/10J) mice infected with Trypanosoma cruzi *(Y strain). Sera obtained during the second week of infection recognized a surface trypomastigote antigen of apparent Mr 80 kDa while displaying complex reactivity to surface epimastigote antigens. Complex trypomastigote antigens recognition was detected around the middle of the third week of infection. No major differences were observed along the infection, among the three strains of mice, neither in the patterns of surface antigen recognition by sera, nor in the titres of antibodies against blood trypomastigotes (lytic antibodies), tissue culture trypomastigotes or epimastigotes. On immunoblot analysis, however, IgG of the resistant strain displayed the most complex array of specificities against both trypo and epimastigote antigens, followed by the susceptible strain. IgM antibodies exhibited a more restricted antigen reactivity, in the three mouse strains studied. Balb/c sera (IgG and IgM) showed the least complex patterns of reactivity to antigens in the range of 30 kDa to 80 kDa. The onset of reactivity in the serum to trypomastigote surface antigens was also dependent on the parasite load to which the experimental animal was subjected.*

Key words: *Trypanosoma cruzi* – antibody and host resistance – mouse strains – antigens recognized

Inbred strains and selected lines of mice differ in their susceptibility to infection by *Trypanosoma cruzi*. Multiple and distinct genes control the levels of parasitemia and survival of infected mice (Wrightsman et al., 1982; Andrade et al., 1985) and the early proliferation of the parasite in the vertebrade host (Boyer et al., 1983; Trischmann, 1984).

The relative contributions of the humoral and cell-mediated immunity to the ultimate control of any of those parameters of infection have not yet been established in spite of the well documented protective role of specific antibodies against *T. cruzi* infection (Brener, 1980). In fact, lines of mice selected for high antibody forming capacity against sheep erythrocytes (Kierszenbaum & Howard, 1976) or against *Salmonella tiphymurium* (Corsini et al., 1982) survive significantly longer a challenge with *T. cruzi*, than low antibody responder lines.

Significant differences in *T. cruzi* specific antibody titres between high and low responder lines are observed from the 10th day of infection (Corsini et al., 1982). More recently Zweerink et al. (1985) were unable to detected remarkable differences in the patterns of 35 S-methionine labelled trypomastigote antigens immunoprecipitated by sera from susceptible and resistant inbred mouse strains, obtained after three, five and ten weeks of infection. On the other hand, it was reported that resistant strains display a much more complex pattern of recognition for epimastigote antigens early in infection, than susceptible strains (Zweerink et al., 1985; Grögl & Kuhn, 1985).

The purpose of this work was to analyse the antibody response in inbred mouse strains, which differ in their susceptibility to *T. cruzi* infection. We chose the strains A/Sn, Balb/c and C57B1/10J which are, respectively, suscep-

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tible, moderately susceptible and resistant to the infection (Trischmann, 1986).

Sera were collected during the acute, subacute and chronic phases of infection and their reactivity against trypomastigotes and epimastigotes was analyzed by: (a) antibody titration, (b) immunoprecipitation of surface radioiodinated parasite antigens followed by sodium dodecyl sulfate polycrylamide gel electrophoresis (SDS-PAGE) and (c) immunoblots (IgG and IgM) of antigens transferred to nitrocellulose membranes following SDS-PAGE of whole parasite lysates.

MATERIALS AND METHODS

Infection of mice – Groups of 20 C57B1/10J (B10), Balb/c and A/Sn male mice, bred in our premises, 10-12 weeks of age (20-22 g) were inoculated subcutaneously (upper thigh) with respectively, 500, 20, 20 *T. cruzi* (Y strain) blood trypomastigotes. These inocula were chosen to promote parasitemia peaks of approximately 5×10^5 parasites/ml at the 11 to 12 days post infection (p.i.), for the three strains of mice. Survival of A/Sn infected mice beyond the acute phase was achieved by adding nifurtimox (Lampit-Bayer do Brasil SA, São Paulo), to the drinking water (0.05%) during the first three weeks after infection. All inoculated mice were checked for parasitemia on the day 7 p.i. The 45 days mortality rate for A/Sn infected nifurtimox-treated mice was 20%, while that for Balb/c mice was 5% and for B10 mice 1%. A group of B10 mice received three boosters of 10^3 blood forms by the intraperitoneal route at intervals of 5 days, starting on the 45 day p.i., before being bled on day 60 p.i. All groups of animals were bled from the retroorbital plexus at days 8, 11, 18, 45 and 60 p.i. The sera were pooled, inactivated at 56 °C 1h and kept frozen in aliquots at –20 °C.

Parasites – The Y strain of *T. cruzi* was used throughout. Epimastigotes were grown in LIT medium (Castellani et al., 1967) at 28 °C. The parasites from three days old cultures (98% epimastigotes) were washed three times by centrifugation (800xg, 10 min, 4 °C) with 10 mM sodium phosphate, 150 mM NaCl, pH 7.2 (PBS) before use.

Tissue culture derived trypomastigotes were obtained from monolayers of LLC-MK2 cells and freed of cell debris and transition forms as described (Zingales et al., 1982). The motile trypomastigotes were washed three times by centrifugation (800 xg, 10 min, 4 °C), with Dulbecco's Modified Eagle's Medium (DMEM, Flow Laboratories, Irvine, Scotland) and used in the experiments.

For the lytic assays, bloodstream trypomastigotes were isolated from blood of immunosuppressed mice. Six weeks old male Swiss outbred mice were injected with cyclophosphamide (Enduxan, Abbot Laboratories do Brasil SA, São Paulo), 300 mg/kg body weight, two days prior to intraperitoneal infection with 4×10^4 blood forms. Blood was collected on the 7th day p.i., defibrinated and the parasites separated by differential centrifugation (100xg, 10 min, 4 °C), and washed twice (1000xg, 20 min, 4 °C) with DMEM. Incubation of an aliquot in fresh normal human serum for 30 min at 37 °C without any lysis of the parasites, ensured a control for the absence of membrane bound immunoglobulins on the surface of the parasites.

Serological tests

Lytic antibody assay – The presence of lytic antibodies in the sera of infected mice was assayed by complement-mediated lysis of blood trypomastigotes obtained from outbred immunosuppressed infected mice as described by Krettli & Brener (1982) with minor modifications.

Conventional indirect immunofluorescence The titres of antibodies in the test sera against culture trypomastigotes and epimastigotes were measured by indirect immunofluorescence as described by Camargo (1966) using formalin-fixed parasites and fluorescein-conjugated anti-mouse immunoglobulins (Cappel Laboratories, Malvern, PA).

Dot immunobinding assay – The titres of antibodies against tissue culture trypomastigotes were quantified by an enzymatic dot immunobinding assay as described by De Gaspari et al. (1986b) using Nonidet-P40 (Shell Química SA, São Paulo) lysed parasites as antigens, peroxidase-labelled second antibodies and diaminobenzidine/H_2O_2 as chromogen/substrate.

Polycrilamide gel electrophoresis – Electrophoresis was performed according to Laemmli (1970) either on linear 7.5% to 15.0% (w/v)

gradients (for the Western-blot technique) or on 8% (w/v) polyacrylamide gels, containing 0.1% SDS, (for identification of surface antigens). Molecular weight markers were run in each gel (Alves et al., 1986). The gels containing radioactive samples were fixed, stained, destained, dried and exposed to Kodak X-ray films in the presence of intensifying screens. Short and long exposures were taken of each gel.

Identification of surface antigens

Labelling of parasites – Washed parasites were radioiodinated by Iodogen (1,3,4,6-tetrachloro-3, 6-diphenylglycoluril (Pierce Chemical Co, III, USA). Parasites (10^8 cells) were incubated in 0.5 ml of PBS containing 250 μCi of Na ^{131}I (IPEN, São Paulo, Brasil) at 4 °C for 10 min, in tubes precoated with 20 μg of Iodogen. After labelling the cells were washed twice with cold DMEM.

Lysis and immunoprecipitation of parasites – Labelled, washed parasites (2×10^8) were resuspended in 1 ml of 10 mM Tris-HCl, pH 8.5, 1 mM phenylmethylsulfonyl (PMSF), 1 mM N -tosyl-L-lysine chloromethyl (TLCK), 2.8 units/ml aprotinin, 25 μg/ml leupeptin and 1% (v/v) Nonidet P-40 and subsequently incubated for 10 min at 37 °C. The lysates were centrifuged at 10,000 xg for 30 min at 4 °C and the supernatants were used immediately for immunoprecipitation. In order to minimize non specific immunoprecipitation, lysates were preabsorved first with a 10% (w/v) suspension of heat killed and formalin-fixed *Staphylococcus aureus* (Cowan I strain), prepared according to Kessler (1975), and next by incubation with a pool of normal mouse sera and a 10% (w/v) suspension of *S. aureus*. The supernatant was divided in aliquots (corresponding to 3×10^7 cells), which were incubated overnight at 4 °C with appropriate dilutions of the test sera. The antigen-antibody complexes were further incubated for 30 min at room temperature with 50 μl of a 10% (w/v) suspension of *S. aureus*. Samples were then processed as described by Zingales et al. (1982) and run on 8% (w/v) SDS-polyacrylamide gels. PMSF, TLCK, aprotinin and leupeptin were purchased from Sigma Chemical Co., St. Louis, MO.

Immunoblot assay – Epimastigotes (1×10^8) or tissue culture trypomastigotes (2×10^8) were lysed in sample buffer (62.5 mM Tris-hydrochloride pH 6.8, glycerol 20% (v/v), SDS 2% (v/v), 2-mercaptoethanol 2% (v/v), bromophenolblue 0.01% (w/v) containing 2 mM PMSF and heated at 100 °C for 3 min. After electrophoresis in 7.5%-15% (w/v) gradient SDS-poly-acrylamide slab gels, the samples were transferred to nitrocellulose sheets (Trans-blot cell, Bio Rad Labs, CA) as described by Towbin et al. (1979).

The blots were processed as described by Araujo (1986) using peroxidase labelled anti-mouse IgM or IgG (Cappel Labs, Malvern, PA) as second antibodies and developed with diaminobenzidine/H_2O_2.

RESULTS

Levels of antibodies to T. cruzi – Antibodies to living trypomastigotes (lytic antibodies) and to fixed trypomastigotes or epimastigotes (immunofluorescence) were detected very early in the sera of mice infected with *T. cruzi* (Table). However, throughout the infection period, even the highly sensitive dot-immunobinding assay as well as the other serological techniques used, failed to disclose any significant differences in the titres of anti-*T. cruzi* serum antibodies among the susceptible and resistant mouse strains studied.

Reactivity of sera with trypomastigote antigens – Fig. 1 shows essentially the same patterns of surface trypomastigote antigens recognition by sera from B10, Balb/c and A/Sn infected mice, over the course of infection. Sera collected between days 8 and 11 p.i. recognized weakly an 80 kDa surface antigen. The development of complex antigen recognition by the sera had a rather sudden onset around day 18 p.i., coincident with the decrease in the levels of circulating blood trypomastigotes. Day 18 sera already reacted to surface antigens recognized by chronic phase (60 days p.i.) sera, including the high molecular weight trypomastigote specific surface antigens (83 kDa to 140 kDa, as described by Zingales et al. (1982). It is noteworthy, that a 64 kDa antigen immunoprecipitated by day 18 sera from all strains, was no longer recognized by chronic phase sera. The latter showed increased reactivity to the 70 kDa-110 kDa cluster of antigens as compared with day 18 sera.

TABLE

Antibody levels in the sera of C57B1/10J, Balb/c and A/Sn mice infected with *Trypanosoma cruzi*

Mouse strain	Day after infection	% Lysis of bloodstream trypomastigotes	Reciprocal titre of serum dilution assayed on			
			Epimastigotes	Tissue culture trypomastigotes		
			Immunofluorescence Total Ig	Immunofluorescence IgM	IgG	Dot Immunobinding assay IgG
C57B1/10J (500)[a]	8	Neg[b]	Neg	Neg	Neg	5
	11	25	Neg	5	Neg	80
	18	65	5	80	20	320
	45	94	40	40	40	320
	60	90	160	40	160	320
	60[c]	97	160	80	320	320
Balb/c (20)[a]	8	Neg	Neg	Neg	Neg	5
	11	35	Neg	5	Neg	40
	18	67	10	80	40	80
	45	89	40	160	80	320
	60	90	40	40	160	320
A/Sn (20)[a]	8	Neg	Neg	Neg	Neg	5
	11	36	Neg	5	10	40
	18	78	40	20	80	160
	45	85	80	40	320	640
	60	90	80	40	320	640

a – Number of *T. cruzi* inoculated by the subcutaneous route. A/Sn mice were treated with nifurtimox.
b – Neg. negative denotes values which were in the range of those obtained for sera of normal non infected mice tested as controls in all assays: percentage of lysis below 10% and titres in the other assays below 1/2.
c – Blood collected from a group of C57B1/10J mice which received three i.p. boosters with 10^3 *T. cruzi* at 5 days intervals before being bled on day 60.

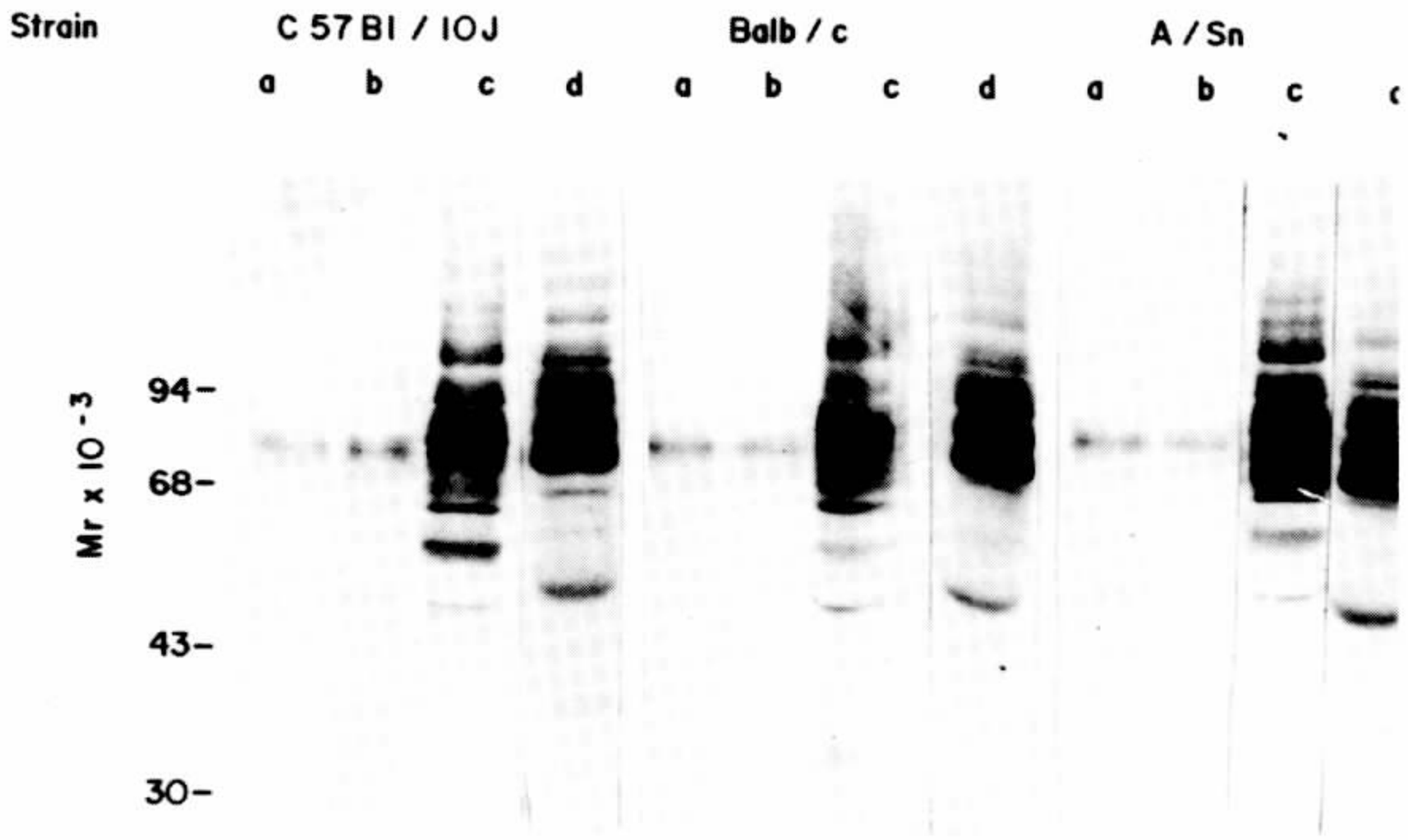

Fig. 1: autoradiograph of SDS-PAGE of ^{131}I labelled trypomastigote surface antigens after immunoprecipitation with pooled sera from infected C57BL/10J, Balb/c and A/Sn mice obtained at different days after infection: 8 days, lane *a;* 11 days, lane *b;* 18 days, lane *c;* 60 days, lane *d*. No antigens were immunoprecipitated with normal mouse sera (data not shown).

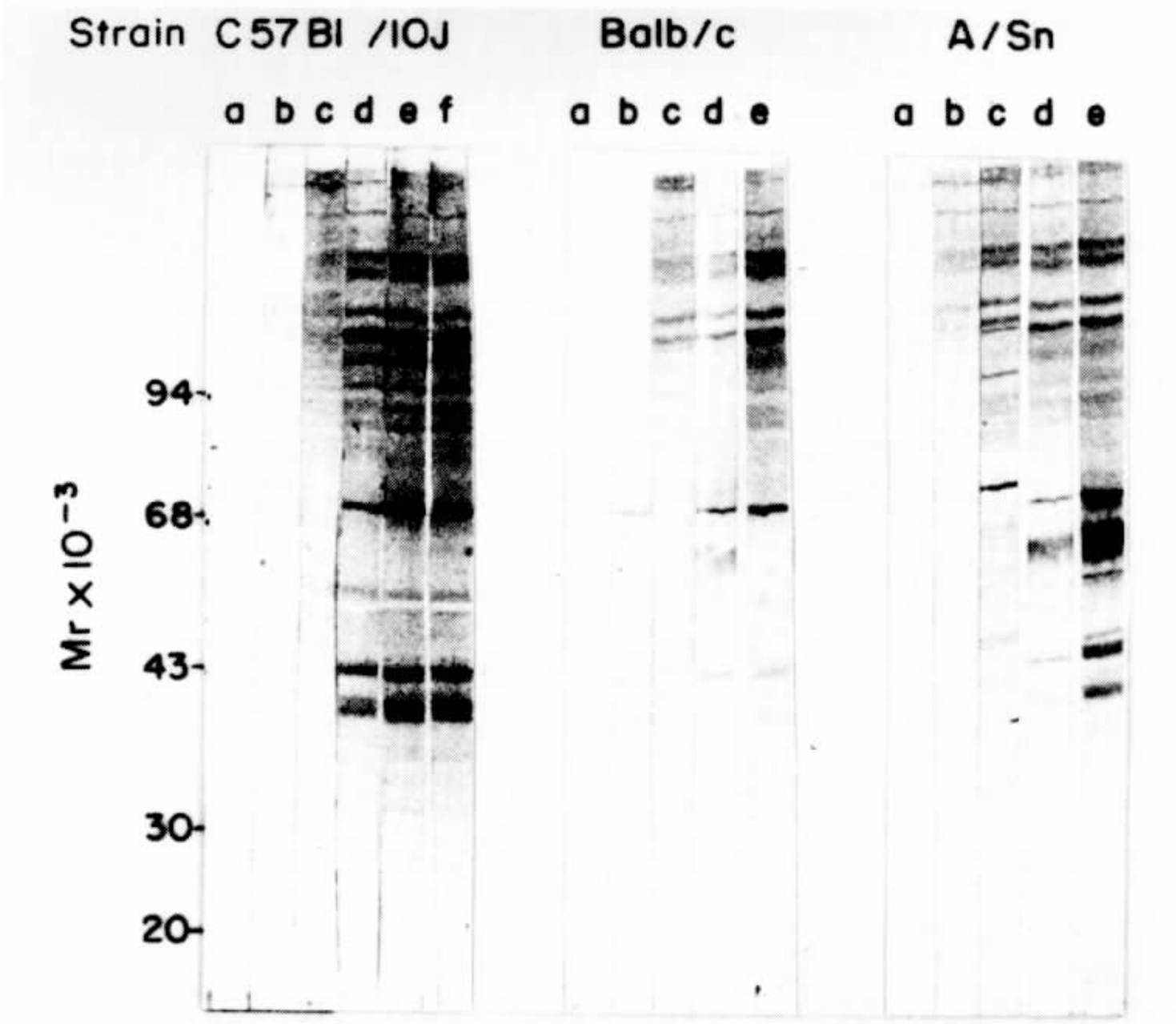

Fig. 2: Western blots of SDS-PAGE of trypomastigote lysates reacted with pooled sera from infected C57BL/10J, Balb/c and A/Sn mice obtained at different days after infection and developed with peroxidase labelled anti-mouse IgG. Lane *a*, 8 days after infection; lane *b*, 11 days; lane *c*, 18 days; lane *d*, 45 days; lane *e*, 60 days; lane *f*, 60 days in previously boostered B10 mice.

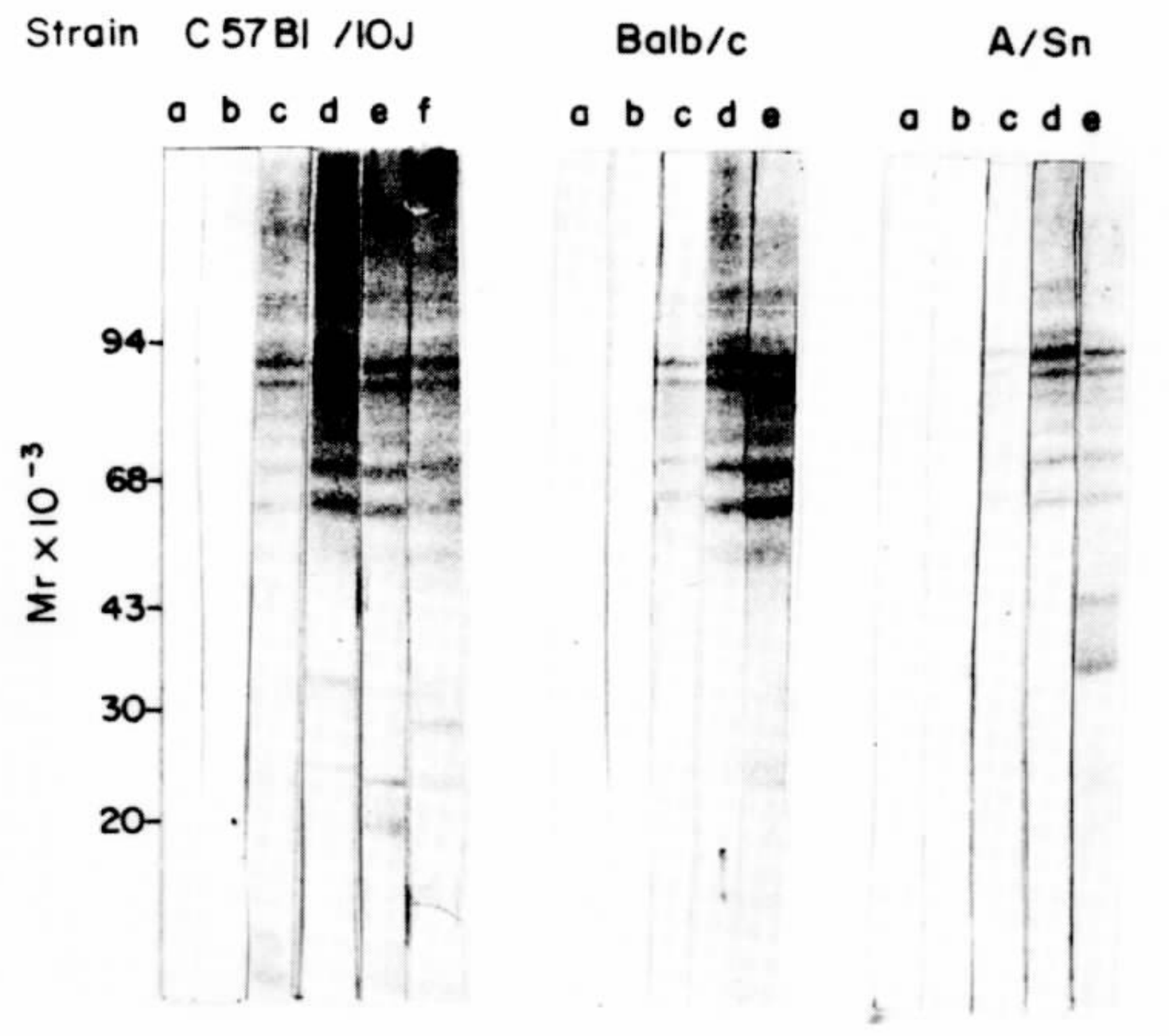

Fig. 3: Western blots of SDS-PAGE of trypomastigote lysates reacted with pooled sera from infected C57BL/10J, Balb/c and A/Sn mice obtained at different days after infection and developed with peroxidase labelled anti-mouse IgM. Lanes *a* through *f* as in Fig. 2.

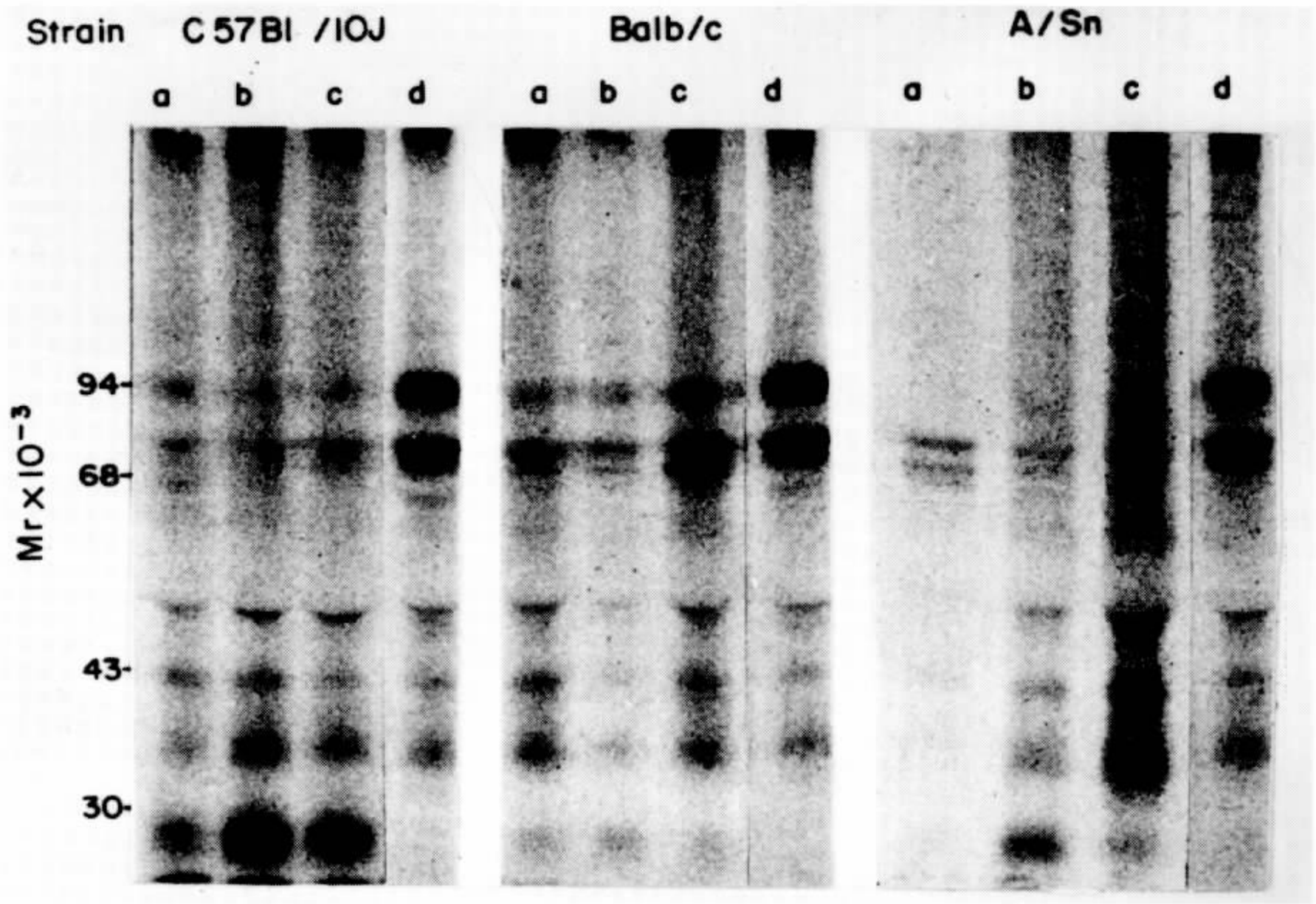

Fig. 4: autoradiograph of SDS-PAGE of ^{131}I labelled epimastigote surface antigens after immunoprecipitation with pooled sera from infected C57BL/10J, Balb/c and A/Sn mice obtained at different days after infection: lanes a through *d* as in Fig. 1.

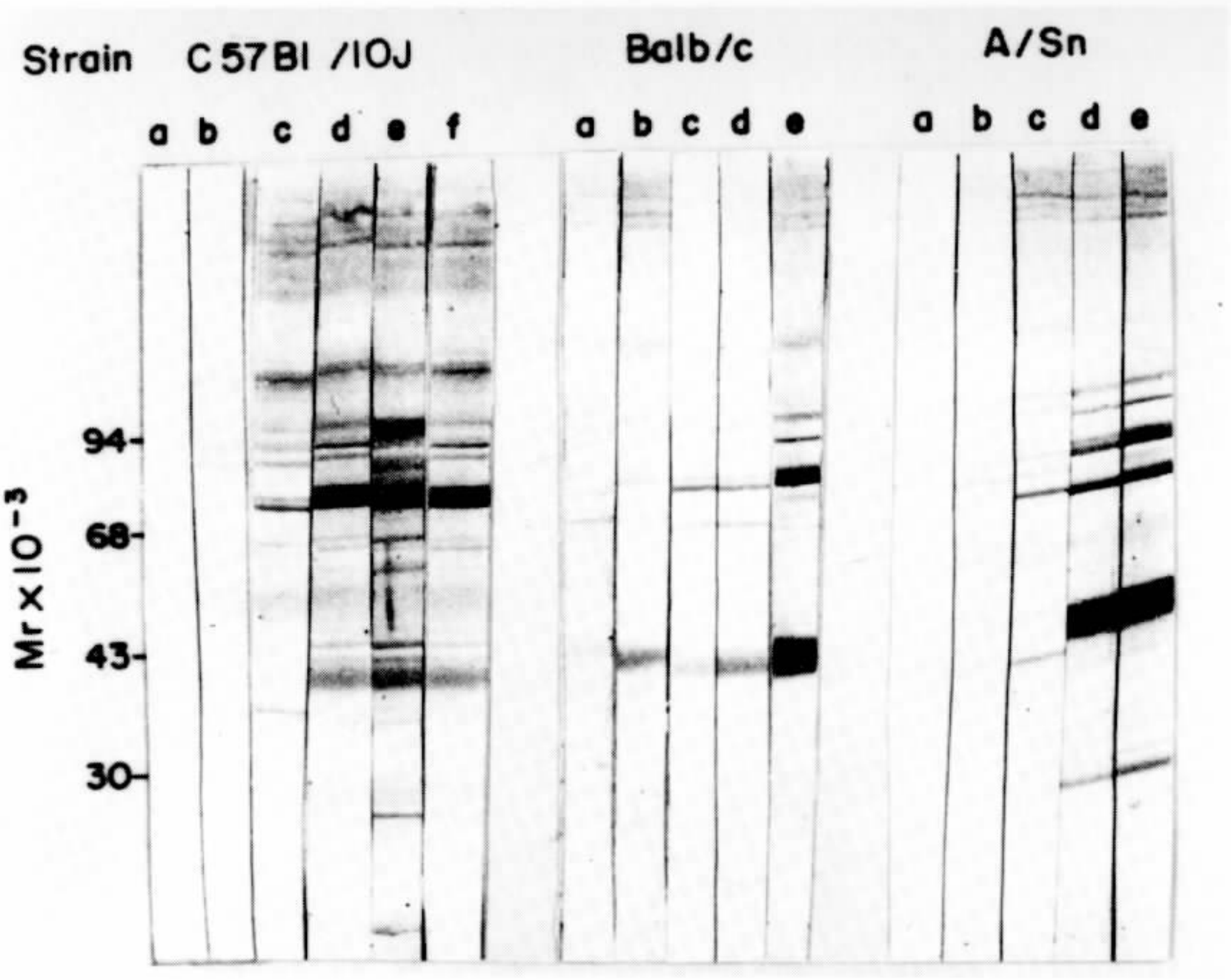

Fig. 5: Western blots of SDS-PAGE of epimastigote lysates reacted with pooled sera from infected C57BL/10J, Balb/c and A/Sn mice obtained at different days after infection and developed with peroxidase labelled anti-mouse IgG. Lanes *a* through *f* as in Fig. 2.

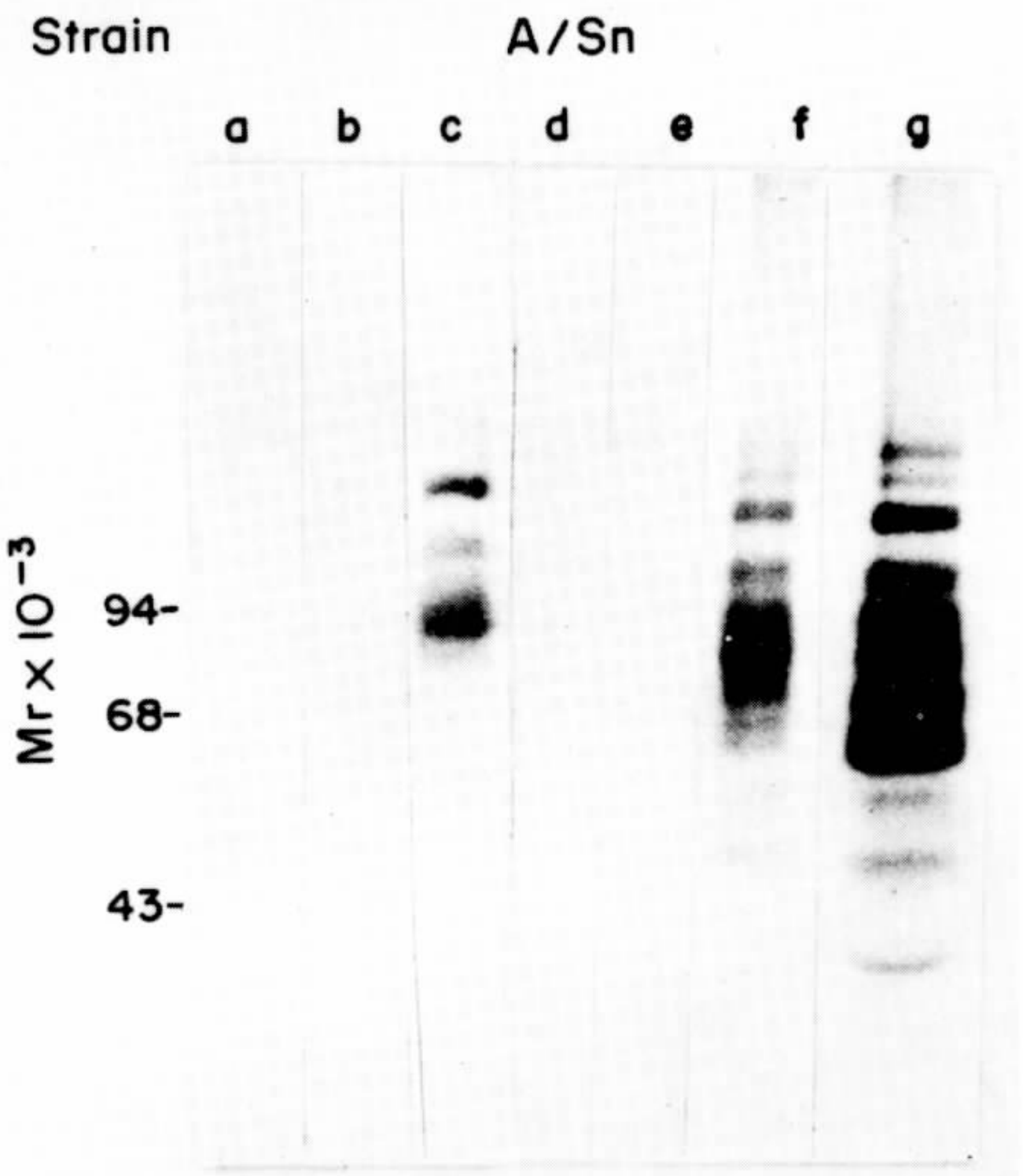

Fig. 6: autoradiograph of SDS-PAGE of ^{131}I labelled trypomastigote surface antigens immunoprecipitated with pooled sera from A/Sn mice infected with 20 *Trypanosoma cruzi:* treated with nifurtimox (lane *d*, 8 days after infection; lane *e*, 11 days; lane *f*, 18 days; lane *g*, 45 days) and left untreated (lane *a*, normal A/Sn sera; lane *b*, 8 days after infection; lane *c*, 11 days after infection).

Figures 2 and 3 show, respectively, the patterns of recognition obtained for IgG and IgM isotypes from infected mouse sera reacted with Western-blots of SDS-PAGE separated trypomastigote lysates.

IgM antibodies reacted with fewer trypomastigote antigens than IgG antibodies in the same sera at any point during the infection. Recognition by IgM became only apparent from the 18th day of infection on, while IgG specific antibodies could already be observed on the 11th day p.i. (Fig. 3 vs Fig. 2). Most polypeptides in the 80 kDa to 130 kDa range were recognized by both IgM and IgG. However, IgM reacted poorly with 70 kDa polypeptide and with polypeptides in the range of 40 kDa-43 kDa and 150 kDa-180 kDa, whereas IgG displayed a strong recognition and definition of these antigens.

The comparative analyses of reactivities of IgM and IgG isotypes among the sera from the three mouse strains showed the most complex spectra of antigen recognition by B10 sera, followed by A/Sn sera. Balb/c sera (IgM and IgG) exhibited a rather restricted reactivity towards antigens in the range of 30 kDa to 80 kDa.

Reactivity of sera with epimastigote antigens – Recognition of surface trypomastigote antigens by sera from the 8th and 11th days p.i. was restricted to an antigen of 80 kDa (cf. Fig. 1). Yet, already on days 8 (B10 and Balb/c strains) and 11 p.i. (all strains), the same sera exhibited very similar spectra of recognition for surface epimastigote antigens as seen for chronic phase sera (Fig. 4).

Detectable antibodies to polypeptides of 90 kDa, 80 kDa, 75 kDa, 53 kDa, 41 kDa, 35 kDa and 25 kDa were found. Sera from chronic phase displayed an enhanced recognition of

the 90 kDa, 80 kDa and 75 kDa surface antigens. No major differences, among the strains, in recognition patterns of antibodies to epimastigote surface antigens were detected along the infection.

The earlier reactivity of mouse sera towards epimastigote antigens as compared to trypomastigote antigens (as shown above for surface labelled antigens), was confirmed in Western-blots of epimastigotes treated with the same sera and developed for IgG (Fig. 5). Polypeptides of 75 kDa and 80 kDa were recognized by sera from all mouse strains from the 8th day p.i. on, and became the most intensely detected antigens by sera from chronic phase B10 mice. Another polypeptide of around 120 kDa was more markedly detected by B10 sera collected from days 8 to 60 p.i. B10 sera displayed the most complex array of antigen recognition against epimastigote antigens in Western-blots when compared to A/Sn sera and Balb/c sera. Although B10 sera reacted with more polypeptides of molecular weight below 70 kDa, A/Sn and Balb/c chronic sera reacted more intensely with antigens of apparent molecular weight in the range of 40 kDa to 50 kDa.

Antigen recognition by sera from mice subjected to a high parasite burden – In order to verify whether a higher antigenic load would antecipate surface antigen recognition by serum antibodies, a lot of A/Sn mice was infected with 20 blood forms and divided in two groups. The group treated with nifurtimox developed its highest parasitemia (5.1 x 10^5/ml) on day 11 p.i. The group left untreated, had maximum parasitemia of 9.8 x 10^5/ml on day 10 p.i. and the mortality rates on day 13 p.i. neared 80%.

Reactivity against trypomastigote surface antigens appeared earlier in the sera of untreated mice (high parasitemia group) than in sera of treated mice (Fig. 6). The former already recognized antigens of Mr 80 kDa, 110 kDa and 130 kDa on the 11th day p.i. Sera of treated mice displayed only weak recognition of 80 kDa polypeptides on days 8 and 11 p.i. and a more complex pattern of reactivity was seen on day 18 p.i. (cf. Fig. 6 vs Fig. 1).

DISCUSSION

The results presented herein emphasize the detection of specific antibody responses very early in the acute phase of *T. cruzi* infection.

As already stressed by Trischmann (1986), antibody independent nonspecific immune mechanisms would control the tissue parasite load during the first cycle of intracellular replication, while the specific immune response is still being stimulated. Antibodies become an important factor for host resistance following the rupture of the parasitized host cells. Thus, antibodies against surface antigens would promote phagocytosis by activated macrophages, cell-mediated cytotoxicity (Krettli, 1982) or inhibit the entry of parasites in non-phagocitic cells (Zingales et al., 1982; Alves et al., 1986). The net effect of an efficient humoral and cell mediated immune response would thus divert infective forms from non-phagocytic susceptible cells and from the circulation. Indeed, macrophages from susceptible and resistant strains of mice exhibit different degrees of activation (Pécora & Barcinski, 1979), possibly related to a more efficient production of activating factors by lymphocytes in the resistant strains (Nogueira et al., 1981). This study failed to uncover any significant differences in the development of anti-*T. cruzi* antibody titres over the course of the infection among susceptible and resistant inbred mouse strains. Corsini et al. (1982) reported higher titres of specific IgG antibodies in (CBA x B10)F1 mice as compared to Biozzi's low antibody responder mice (susceptible). However, the differences in titres never exceeded two log 2 dilutions of the sera, at dilutions below 1/10.

On the other hand, Grögl & Kuhn (1985) found earlier and higher IgM and IgG antibody levels to epimastigote antigens in susceptible (C3H) mice than in C57BL/6J resistant mice. Since both strains were infected with identical inocula, C3H mice were subjected to a several fold higher parasite load. Inasmuch as parasitemia levels are indicative of parasite load, we sought different infective doses of *T. cruzi* which would result in parasitemia peaks of similar magnitude and onset, in order to be able to compare the antibody response among susceptible and resistant strains.

Our own results (Fig. 6) showed that sera from susceptible mice allowed to develop high parasitemias, displayed earlier reactivity against trypomastigote antigens. This observation suggests the importance of the parasite antigenic load in determining the rate of the humoral response.

Susceptible and resistant strains of mice infected with *T. cruzi* did not differ in regard to the onset and patterns of reactivity in their sera against trypomastigote or epimastigote surface antigens. The high molecular weight trypomastigote specific antigens (Zingales et al. 1982) were immunoprecipitated by sera obtained from the third week of infection on (day 18 p.i.). However, sera obtained on the 8th day p.i. (11 for A/Sn sera), already exhibited the full spectra of recognition for surface epimastigote antigens, seen with chronic phase sera, while recognizing only an antigen of apparent Mr 80 kDa on the surface of trypomastigotes. The epimastigote surface antigens immunoprecipitated by sera from the three strains of infected mice showed apparent Mr of 90 kDa, 80 kDa, 75 kDa and 53 kDa. These antigens probably represent glycoproteins sharing common epitopes in epimastigotes and trypomastigotes, as reviewed by Andrews et al. (1984), Snary (1985) and Zingales & Colli (1985). The absence of obvious differences in the patterns of surface antigen recognition by sera from susceptible and resistant mice, studied by immunoprecipitation followed by one dimension SDS-PAGE, does not exclude more subtle differencies in early antigen recognition. Indeed, two dimensional electrophoresis would lead to a better characterization. In addition, in contrast with the results of Zweerink et al. (1985), we found that B10 resistant mice displayed a more complex pattern of serum reactivity against trypomastigote antigens than A/Sn and Balb/c mice. The array of specificities recognized by IgG from all strains become increasingly complex from the 11 day p.i., on. Stronger reactions were observed for several antigens, but the immunoblot patterns remained qualitatively unchanged from the 45 day p.i. on.

The reactivity of IgM antibodies to trypomastigote antigens was less complex than that obtained for IgG in the same sera for the three mouse strains studied with no obvious increment in recognition over the course of infection.

The results are consistent with the lower titres of IgM as compared with IgG antibodies after the third week of infection (Table) and the lower affinity of IgM antibodies expected in a primary response at the beggining of the infection.

The analysis of reactivity of the sera (IgG) against epimastigote antigens by the immunoblot technique confirms the very early appearance of epimastigote reactive antibodies during infection, already described by Grögl & Kuhn (1985). However, we again detected a more complex pattern of recognition for B10 mice sera when compared to A/Sn and Balb/c, analogous to our results using trypomastigote antigens. The 40 kDa – 50 kDa polypeptides recognized by sera from all strains (more intensely so by A/Sn and Balb/c) possibly include the 51 kDa glycoprotein described by Scharfstein et al. (1986) as the uncleaved form of the 25 kDa antigen.

The discrepancies between our results and those of Zweerink et al. (1985) may be explained by their different methodological approach (metabolic labelling by 35 S-methionine, discontinous SDS-PAGE and the times chosen to collect the serum samples).

Moreover, our own results (De Gaspari et al., 1986b) indicate that antibodies recovered from the surface of blood trypomastigotes on the 8th day of infection react with several trypomastigote surface antigens although reactivity of the serum obtained at the same time is rather restricted. This finding adds further complexity to the analysis of antigen recognition by specific antibodies during the acute phase of infection.

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