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Enzyme-linked immunosorbent assay for detection of retroviral gp70 and gp70-anti-gp70 immune complexes in sera from SLE mice

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SUMMARY

Since a retroviral envelope glycoprotein, gp70, present in sera is prominently involved in the pathogenesis of murine systemic lupus erythematosus (SLE), the detection of gp70-anti-gp70 immune complexes (gp70 IC) is particularly useful for the study of murine SLE. To facilitate the detection of gp70 and gp70 IC, we have developed a simple and sensitive enzyme-linked immunosorbent assay (ELISA). Using an affinity column coupled with whole mouse serum proteins containing serum gp70 or with Rauscher murine leukaemia virus (MuLV), antibodies specific to serum gp70 or to Rauscher MuLV gp70 were purified from hyperimmune goat anti-Rauscher MuLV gp70 antisera. Only affinity-purified anti-serum gp70 fraction, but not anti-Rauscher MuLV gp70 fraction, was able to detect serum gp70 efficiently in the ELISA, because only a minor fraction of goat anti-Rauscher MuLV gp70 antibodies is cross-reacting with serum gp70. This procedure could be applied to other antigen-antibody systems, in which only antibodies to heterologous cross-reacting antigens are available, to detect free and antibody-complexed antigens in pathological sera.

Keywords retroviral gp70 antigen gp70 immune complex SLE ELISA

INTRODUCTION

The major envelope glycoprotein, gp70, of murine retrovirus is present in the serum of virtually all mice (Yoshiki *et al.*, 1974; Strand & August, 1976; Lerner *et al.*, 1976). The gp70 circulates free from any association with viral particles and its expression is independent of the activation of endogenous retroviruses. More recently, the gp70 has been demonstrated to behave as an acute phase reactant and is synthesized by hepatic parenchymal cells like many other serum proteins (Hara, Izui & Dixon, 1982). Thus, serum gp70 should be considered as an autologous protein.

It has been made clear that serum gp70 participates significantly in the formation of immune complexes (IC) and contributes to the pathogenesis of murine systemic lupus erythematosus (SLE) (Yoshiki *et al.*, 1974; Andrews *et al.*, 1978; Izui *et al.*, 1979). The apparent deposition of IC containing gp70 has been demonstrated not only in diseased glomeruli but also in the walls of blood vessels associated with degenerative vascular disease and myocardial infarction (Accini & Dixon, 1979). The pathogenic significance of gp70-anti-gp70 (IC) (gp70 IC) was further supported by genetic studies in (NZB × NZW) × NZW or NZB backcross mice (Nakai *et al.*, 1980; Maruyama *et al.*, 1983) and in (NZB × NZW)F_2 mice (Izui *et al.*, 1981b). Clearly, gp70 IC is a useful and predictive indicator of murine SLE.

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Since the detection of gp70 and gp70 IC requires the highly purified gp70 antigen from murine leukaemia virus for the radioimmunoassay (RIA), this assay has not been widely used, although the measurement of this parameter is extremely useful in the studies of murine SLE. Therefore, in the present study, we have developed an enzyme-linked immunosorbent assay (ELISA) using affinity purified antibodies to serum gp70 for the detection of gp70 and gp70 IC.

MATERIALS AND METHODS

Mice

NZB, NZW, BXSB, C57BL/6 (B6), DBA/2 and BALB/c mice were purchased from Olac Laboratories, Oxon, England. (NZB × NZW)F_1 hybrid (NZB × W) mice were obtained by local breeding. C57BL/6-G_{IX}^+(B6-G_{IX}^+) mice were provided by Dr E.A. Boyse, Memorial Sloan-Kettering Cancer Center, New York, USA. Mice were bled from the retro-orbital plexus, and their sera were stored at −20°C until use.

Antisera to retroviral gp70 and retrovirus

Goat anti-Rauscher murine leukaemia virus (MuLV) gp70 and anti-feline leukaemia virus (FLV) antisera were obtained from the National Cancer Institute, Bethesda, Maryland, USA. Anti-Rauscher MuLV gp70 antibodies were affinity purified on a CNBr-activated Sepharose 4B column coupled with Rauscher MuLV.

Preparation of affinity column coupled with total serum proteins
Sera enriched with gp70 were first prepared in 2-month-old NZW mice by injecting 50 μg bacterial lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4 (Calbiochem-Behring Corp., San Diego, California, USA), since LPS markedly elevated serum levels of gp70 due to an enhanced production of gp70 by hepatic cells (Hara *et al.*, 1982). Pooled sera collected 24 h after the LPS injection were directly coupled on CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden).

Affinity purification of anti-serum gp70 antibodies
Goat anti-Rauscher MuLV gp70 were directly applied to the CNBr-activated Sepharose 4B coupled with whole serum proteins containing a relatively high concentration of gp70. Bound anti-serum gp70 antibodies were eluted with 1 M KSCN and dialysed extensively against 0·01 M phosphate-buffered saline, pH 7·4 (PBS).

Conjugation of antibodies with alkaline phosphatase
The anti-gp70 and anti-IgG antibodies were coupled with alkaline phosphatase (Sigma Chemical Co., St Louis, Missouri, USA) by the glutaraldehyde method (Engvall & Perlmann, 1972).

ELISA for serum gp70 and gp70 IC
Polystyrene microtitre plates (Flow Laboratories, Inc., McLean, Virgina, USA) were coated with affinity purified goat anti-serum gp70 or normal goat IgG (Miles Laboratories, Inc., Elkhart, Indiana, USA) (10 μg/ml) in borate-buffered saline for 3 h at 37°C, followed by washing with PBS. After incubation with 0·5% bovine serum albumin (BSA) in PBS for 1 h at room temperature, samples diluted with 2% BSA in PBS containing 0·05% Tween 20 were added to the wells for an overnight incubation at 4°C. After washing, wells were incubated with alkaline phosphatase-conjugated goat anti-serum gp70 antibodies for the detection of gp70 or with alkaline phosphatase-conjugated goat anti-mouse γ-chain specific antibody (Cappel Laboratories, Cochranville, Pennsylvania, USA) for the detection of gp70 IC. After incubation for 5 h at 4°C, enzyme substrate (paranitrophenyl phosphate, Sigma Chemical Co.) was added in 0·01 M diethanolamine solution, pH 9·8. The colour intensity was measured at OD 405 nm with Titertek Multiskan (Flow Laboratories, Inc.).

RIA for serum gp70
Concentrations of gp70 in sera were determined by their capacity to inhibit the binding of goat anti-FLV antibodies to ^{125}I-labelled gp70 from Rauscher MuLV. The details of this RIA were described previously (Izui *et al.*, 1979).

Sucrose density gradient ultracentrifugation
Serum samples of 0·05 ml were layered on 5–20% (w/v) linear sucrose gradient in PBS and centrifuged at 80,000 *g* for 18 h at 4°C in a SW 60 rotor in a Beckman 175 ultracentrifuge. Positions of IgG and IgM were established by using radioactive markers. Gradients were divided into 12 fractions.

RESULTS

Development of an ELISA for detection of serum retroviral gp70 antigen

In initial experiments, in order to develop an ELISA for the detection of serum retroviral gp70 antigen, the attempt was made to use an IgG fraction of affinity-purified goat anti-Rauscher MuLV gp70 antibodies for coating the microtitre plates and developing the assay. After incubation with a serum pool from NZB mice, which express relatively large amounts (~50 μg/ml) of gp70 in sera, the assay was developed by alkaline phosphatase-conjugated anti-Rauscher MuLV gp70 antibodies used for coating the plates. Although this assay was very efficient at detecting Rauscher MuLV gp70, it was not successful for serum retroviral gp70. This failure is apparently because goat anti-Rauscher MuLV gp70 antibodies contain only limited amounts of antibodies cross-reactive with murine serum gp70, which has immunological characteristics similar to those of NZB xenotropic virus (Elder *et al.*, 1977; Izui *et al.*, 1981a).

To enhance the sensitivity of the detection of serum gp70, anti-gp70 antibodies cross-reacting with serum gp70 were affinity-purified from goat anti-Rauscher MuLV gp70 antisera by a column coupled with serum proteins containing high concentrations of gp70. Using these affinity-purified anti-gp70 antibodies for the coating and the development, a significant binding of serum gp70 from NZB mice was observed (Fig. 1). Figure 1 also shows the binding curve obtained by sera from B6-G_{IX}^+ and B6-G_{IX}^- congenic mice. A serum pool from B6-G_{IX}^+ mice exhibited approximately five times higher binding than that of congenic G_{IX}^- counterparts, and these results were comparable to those obtained by the RIA (Hara *et al.*, 1981). It should be noted that there was no binding at all when serum samples were incubated with microtitre plates coated with normal goat IgG instead of anti-gp70 antibodies.

The sensitivity of ELISA for the detection of serum gp70 was superior to that of RIA (Fig. 2). The limit of the detection by ELISA and RIA was around 5 ng/ml and 20 ng/ml, respectively. To confirm that the results of the ELISA were comparable to those of RIA, serum levels of gp70 were determined in murine

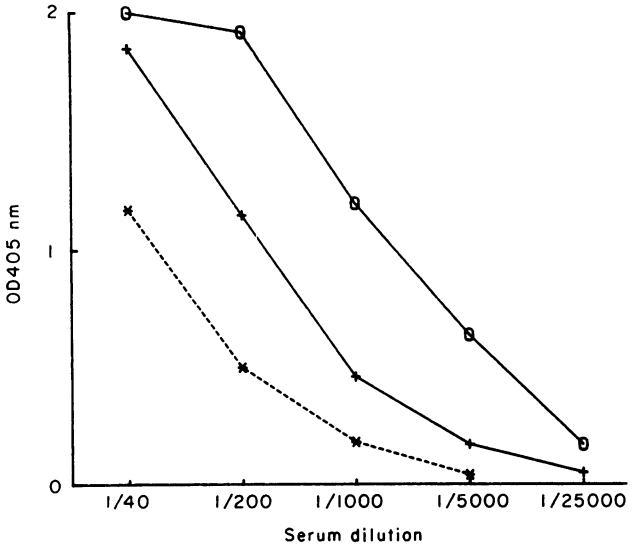

Fig. 1. Detection of gp70 in pooled serum from NZB (O——O), B6-G_{IX}^+ (+——+) or B6-G_{IX}^- (*-----*) mice by ELISA. Various dilutions of sera were first incubated with microtitre plates coated with affinity purified anti-serum gp70 antibodies. Then, the bound gp70 was revealed by alkaline phosphatase-labelled anti-serum gp70 conjugates.

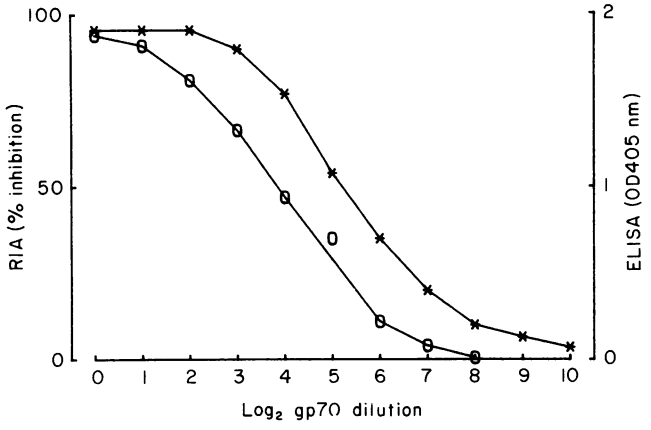

Fig. 2. Detection of serum gp70 in NZB mice by RIA (O——O) or ELISA (*——*). A 1/20 diluted NZB serum pool (2·5 μg of gp70 per ml) was serially diluted in PBS containing 2% BSA and 0·05% Tween 20, and tested either in the RIA or in the ELISA.

Table 1. Comparison of serum gp70 levels determined by ELISA and RIA in various strains of mice

	gp70* (μg/ml)	
Mice	ELISA	RIA
NZB	68·7	59·0
NZW	64·2	53·6
DBA/2	30·4	27·0
BXSB	12·4	10·2
B6-G_{Ix}+	10·6	8·6
B6-G_{Ix}−	2·2	1·8
BALB/c	1·2	0·8

* gp70 levels in pooled sera from 2-month-old female mice (5–7 mice) were determined by ELISA or RIA.

strains whose sera contain high (> 10 μg/ml) or low (< 5 μg/ml) amounts of gp70, by both assays with the standard curve established by a serum pool containing known amounts of gp70. Results obtained by ELISA were essentially identical to those by RIA in all the strains tested (Table 1).

Development of an ELISA for detection of gp70 IC in sera

The presence of gp70 IC in sera was detected by the similar ELISA using alkaline phosphatase-labelled anti-mouse γ-chain antibodies instead of anti-gp70 antibodies. Serially diluted pooled sera from 6-week-old and 8-month-old NZB × W female mice were first incubated with microtitre wells coated with affinity purified anti-serum gp70 antibodies. Then, the assay was developed with enzyme-labelled anti-mouse γ-chain antibodies. In this assay, only the serum pool from 8-month-old but not from 6-week-old female mice exhibited a significant binding by anti-mouse γ-chain antibodies (Fig. 3). It should be noted that an essentially identical binding curve was obtained by both sera when the assay was developed with enzyme-labelled anti-gp70

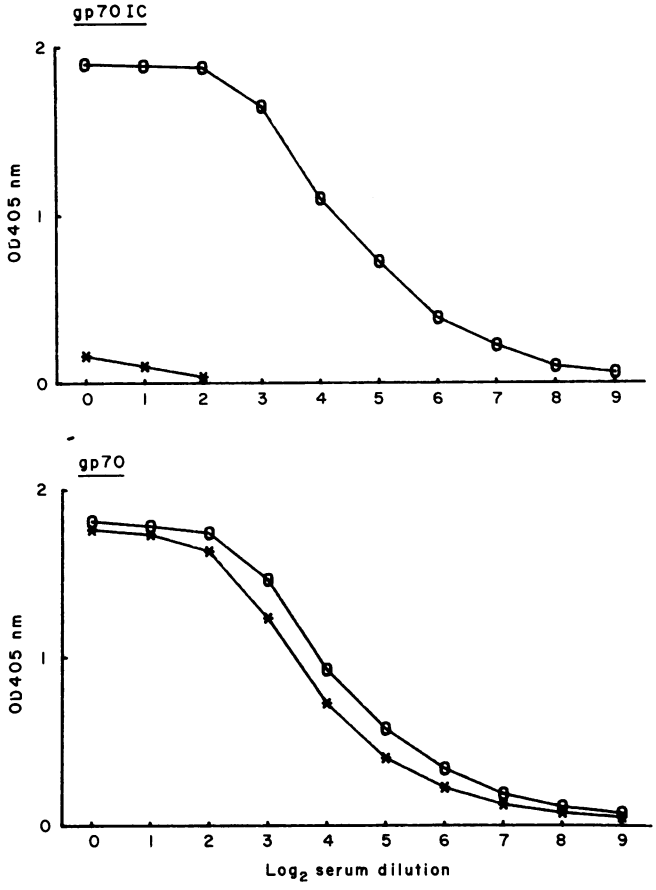

Fig. 3. Detection of gp70 IC and gp70 in pooled serum from 6-week-old (*——*) or 8-month-old NZB × W female mice (O——O) by ELISA. 1/100 diluted pooled sera were serially diluted and then incubated with wells coated with anti-serum gp70. The assay was developed with either enzyme-labelled anti-mouse γ-chain antibodies for the detection of gp70 IC or goat anti-serum gp70 antibodies for the detection of gp70.

antibodies. When sera were incubated with normal goat IgG-coated plates, a minimal binding (OD405 values: ~0·100) was observed by enzyme-labelled anti-γ-chain, but not at all by anti-gp70 conjugates.

These same pooled sera were also compared in terms of the sedimentation of gp70 molecules. After ultracentrifugation in sucrose density gradients, the presence of gp70 was measured in each of 12 serial gradient fractions by the ELISA for gp70. In the pooled serum from 6-week-old NZB × W female mice, gp70 appeared in or near 5S region containing the free gp70 molecule (Fig. 4). In contrast, when the pooled serum from 8-month-old NZB × W female mice were ultracentrifuged, gp70 formed a broader band; substantial amounts of gp70 were detected in fractions heavier than 7S IgG. Notably, these results are comparable to those obtained by RIA (data not shown).

When individual fractions from these two gradients were analysed for the presence of IgG complexed with gp70 by the ELISA for gp70 IC, none of fractions from 6-week-old NZB × W female mice exhibited significant binding by anti-mouse γ-chain antibodies (Fig. 4). In contrast, the presence of gp70 IC was detected in all the fractions heavier than 7S IgG in the pooled serum from 8-month-old NZB × W female mice.

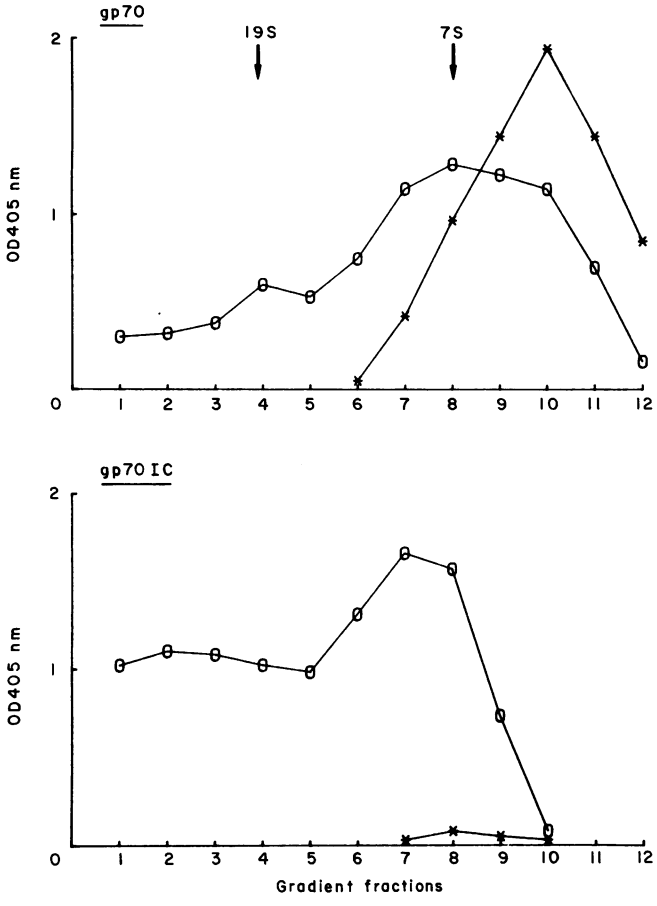

Fig. 4. Detection of gp70 IC in sucrose density gradient fractions of pooled serum from 6-week-old (*——*) or 8-month-old (O——O) NZB × W female mice. Each gradient fraction diluted 1/10 was subjected to gp70 and gp70 IC ELISA. The position of markers is indicated by the arrows.

Detection of gp70 IC by the ELISA in sera from SLE-prone NZB × W mice

Sera from 2-month-old and 8-month-old NZB × W, 8-month-old NZW and 8-month-old B6 female mice were individually analysed for the presence of gp70 IC by the ELISA. Since we noticed that some pathological sera occasionally exhibited weak, but significant, binding goat IgG-coated plates, presumably due to the hypergammaglobulinemia, sera diluted at 1:1,000 were incubated with either anti-gp70 or normal goat IgG-coated plates. Then, the assay was developed with alkaline phosphatase-labelled anti-mouse γ-chain antibodies. Results are expressed as OD_{405} after background values obtained by normal goat IgG plates were subtracted. Although sera for 8-month-old NZB × W females showed a minimal binding to normal goat IgG-plates with mean OD_{405} values of 0·175, the binding of their serum IgG to anti-gp70 plates was highly significantly elevated (Table 2). Mean of their specific binding to anti-gp70-coated plates was 1·073, confirming that substantial amounts of serum gp70 in 8-month-old NZB × W females were complexed with anti-gp70 antibodies as described previously (Izui *et al.*, 1979). In contrast, no detectable amounts of circulating gp70 were complexed with anti-gp70 antibodies in 2-month-old NZB × W, 8-month-old NZW and 8-month-old B6 female mice, since none of their serum IgG exhibited significant binding to anti-gp70-coated plates (Table 2).

Table 2. Detection of gp70 IC by the ELISA in sera from 8-month-old NZB × W female mice

Strain	Age (months)	gp70 IC ELISA* Anti-gp70	Goat IgG	Specific binding
NZB × W	2 (8)†	0·113 ± 0·041	0·100 ± 0·040	0·013 ± 0·040
	8 (12)	1·248 ± 0·334	0·175 ± 0·087	1·073 ± 0·298
NZW	8 (8)	0·056 ± 0·031	0·044 ± 0·010	0·012 ± 0·016
B6	8 (8)	0·047 ± 0·019	0·046 ± 0·014	0·001 ± 0·012

* gp70 IC ELISA was developed with alkaline phosphatase-labelled goat anti-mouse γ-chain antibodies. Results are expressed as the OD405 nm (mean ± 1 s.d.).

† Number of mice tested.

DISCUSSION

Virtually all strains of mice have in their sera relatively large amounts of retroviral gp70, but only SLE-prone mice spontaneously develop antibodies directed against the serum gp70. Serum gp70 complexed to anti-gp70 antibodies has been shown under different experimental conditions to be prominently involved in the renal disease of murine SLE (Yoshiki *et al.*, 1974; Nakai *et al.*, 1980; Izui *et al.*, 1980; 1981b). Clearly, gp70 IC is a useful and predictive indicator of murine SLE.

In the present study, we have developed an ELISA using affinity purified antibodies to serum gp70 for the detection of gp70 antigen and gp70 IC. Studies on sera from various strains of mice including the G_{1X} congenic strain and on sucrose gradient fractions have demonstrated that the ELISA detects specifically serum gp70 antigen and its complexes, and these results are comparable to those of the RIA (Yoshiki *et al.*, 1974; Lerner *et al.*, 1976; Izui *et al.*, 1979; Hara *et al.*, 1981). Clearly, the ELISA is simpler and more sensitive to detect serum gp70 and gp70 IC than the RIA, which is based on the serum inhibitory activity of the binding of goat anti-feline leukaemia virus antibodies to ^{125}I-labelled gp70 from Rauscher MuLV.

Our ELISA for the detection of gp70 and gp70 IC has additional advantages compared with the RIA. First, obviously, one does not need the radiolabelled purified gp70 antigen. This is, in fact, the major problem of gp70 detection by the RIA. gp70 is a very labile glycoprotein and we have always noticed considerable degradation of gp70 just after iodination, so radiolabelled gp70 has to be fractionated by Sephadex column to purify intact labelled gp70 molecule before use. Instead, the ELISA requires the affinity purification of anti-gp70 antibody and its conjugation with alkaline phosphatase, whose procedures are relatively simple and have been routinely carried out in many laboratories. Second, gp70 determination by the ELISA is not affected by the presence of free anti-Rauscher gp70 antibodies occasionally present in serum from SLE-prone NZB × W and MRL-*lpr*/*lpr* mice (Izui *et al.*, 1979). Third, the detection of gp70 IC by the ELISA does not require the treatment of sera, e.g. absorption with *Staphylococcus aureus* protein A (Izui *et al.*, 1980) or precipitation with polyethylene

glycol (Maruyama *et al.*, 1983) to separate gp70 IC from free gp70 for the quantification of gp70 IC by the RIA. Finally, the ELISA would allow us to analyse the isotype and IgG subclasses of anti-gp70 antibodies involved in the formation of gp70 IC, which is not possible by the RIA.

The only problem that one has to be cautious about in the ELISA is the non-specific binding of murine IgG to goat IgG. At least for the detection of gp70 antigen, we have never found any non-specific binding on normal goat IgG plates when developed by enzyme-labelled anti-gp70 antibodies. However, sera from aged NZB × W mice occasionally exhibited a weak but significant binding to normal goat IgG plates when developed by enzyme-labelled goat anti-mouse γ-chain antibodies, which is most probably a result of non-specific Fc-Fc interaction of IgG. Therefore, it would be better to control the specificity for the detection of gp70 IC in the parallel assay using normal goat IgG-coated plates.

It should be noted that only the IgG fraction purified from goat anti-Rauscher MuLV gp70 antisera on the affinity column coupled with serum gp70 efficiently detect serum gp70 in the ELISA, while the IgG fraction similarly purified, but using the column coupled with Rauscher MuLV fails to detect serum gp70. This is because only a minor portion of goat anti-Rauscher MuLV gp70 antibodies has the cross-reactivity to serum gp70, which immunologically and structurally resembles NZB-type xenotropic viral gp70 (Elder *et al.*, 1977; Izui *et al.*, 1981a). This indicates that if antibodies to heterologous antigens are available, one could develop a solid-phase assay using antibodies purified from affinity columns coupled with crude antigen preparations. In fact, we have recently succeeded in developing an ELISA for the detection of murine haptoglobin, by using cross-reactive antibodies to murine haptoglobin purified from commercial goat anti-human haptoglobin antisera on an affinity column coupled with whole mouse serum proteins. Clearly, one could apply this procedure for other antigen-antibody systems to detect free or antibody-complexed antigens in various pathological sera.

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