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Use of Protein A-Treated Sera in Unmasking Herpes Simplex Virus Type 1 (HSV-1) Immunoglobulin A and Identifying HSV-1 Immunoglobulin G as the Predominant Neutralizing Antibody

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Treatment of human sera with protein A reduced the amounts of immunoglobulin G (IgG), IgA, and IgM detected by radial immunodiffusion. This treatment also decreased the amount of herpes-specific IgG and IgM detected by radioimmunoassay, whereas it increased and even unmasked the amount of herpes-specific IgA detected. Comparison of protein A-treated sera with untreated sera indicated that herpes simplex virus type 1 IgG was responsible for more than 92 to 99% of the serum neutralizing activity.

High titers of serum antibodies to certain infectious agents appear to be correlated with protection from disease caused by these agents; this has been the historical basis for immunization programs. However, this is not true for all agents, and resistance to infection with certain organisms bears no known relationship to the titer of serum antibody. For example, repeated episodes (2, 3, 6, 9) of oral herpes lesions occur in subjects with high titers of serum neutralizing antibody to herpes simplex virus (HSV).

Tokumaru (13) suggested than an imbalance in the ability to produce HSV-specific immunoglobulin G (IgG), IgA, and IgM may be responsible for recurrent herpes lesions. He reported that complications of primary herpes infections and recurrent episodes appeared to be related to a deficiency of herpes-specific serum IgA. Kurtz (4), by using the complement fixation test, reported that in primary disease the herpes-specific IgM and IgG appeared at the same time and that HSV-1 IgM lasted for at least 8 weeks.

Radioimmunoassay (RIA) is a more sensitive system for the detection of viral antibodies than either the neutralization test used by Tokumaru (13) or the complement fixation test used by Kurtz (4). The present study describes an RIA to detect serum HSV-1 class-specific antibodies. This testing system was applied to sera treated with protein A to remove IgG, so that HSV-1 IgA and HSV-1 IgM might be more accurately detected. Neutralization tests were also performed to determine which antibody class is responsible for serum neutralizing activity.

MATERIALS AND METHODS

Specimens. Human volunteers were sampled for blood specimens; serum was separated from whole blood by centrifugation and stored at −70°C until used. Only sera with HSV antibodies, as detected by RIA, were used in this study.

Virus and cell cultures. The Patton strain of HSV-1 was kindly provided by F. Rapp, Pennsylvania State University College of Medicine, Hershey, Pa. Cultures of human amnion or human fetal lung cells were grown in Eagle basal medium (Auto Pow BME, Flow Laboratories, Inc., Rockville, Md.) with 10% fetal calf serum, 0.11% $NaHCO_3$, 0.03% *l*-glutamine, and 20 μg of neomycin per ml (hereafter known as medium).

Preparation of HSV-1 antigen. Human amnion or human fetal lung cells were inoculated with HSV-1 and adsorbed for 1 h at room temperature. The inoculum was removed; the culture was refed with medium and incubated at 37°C until a 4+ cytopathogenic effect was seen. Cells were scraped from the flask and centrifuged at 1,000 rpm for 3 min. The cells were washed twice in phosphate-buffered saline (PBS) and then frozen at −70°C. The pellet was thawed and suspended in 10 ml of PBS, and five samples of 2 ml each were prepared and frozen at −70°C. For use as antigen, 4 ml of PBS was added to one sample, and the mixture was treated in a sonicator (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) at a setting of 7 for 20 s. Uninoculated cell cultures treated the same way were used as control antigens.

Chloramine-T procedure for ^{125}I labeling of antiglobulins. Purified rabbit antiserum to human globulin or rabbit antiserum to goat globulin was iodinated by the procedure of McConahey and Dixon (7). The dilution to be used was determined by testing various dilutions of the labeled antiglobulin with constant amounts of other reagents in the test systems.

RIA procedure. The RIA procedure described previously by Smith et al. (12) and Patterson and Smith (8) was followed. The magnetic system of transferring beads, reported by Smith and Gehle (11), was used throughout all RIA procedures.

Polycarbonate-coated beads were incubated with

the appropriate antigen or control for 1 h at 4°C. The excess antigen solution was then removed, and the sensitized beads were soaked in 1% bovine serum albumin contained in a solution of PBS and 0.1% sodium azide (hereafter known as diluent) for 5 min. A 1:200 dilution of serum was made in diluent, and additional threefold serial dilutions were made. The beads were placed in appropriate dilutions of serum and held overnight at 4°C. They were used to detect either total or class-specific antibody to HSV-1.

To detect total HSV antibody, the beads were washed in water and incubated in ^{125}I-labeled rabbit antiserum to human globulin for 3 h at room temperature. The beads were washed again, placed in tubes, and counted in a Packard Auto-Gamma counter.

Highly potent and purified goat anti-human IgG, IgA, IgM, and rabbit antiserum to goat globulin, to be used in the detection of HSV-1 class-specific antibodies, were kindly supplied by Richard Keightley, Department of Pediatrics, University of Texas Health Science Center at San Antonio. The beads, which had been incubated in serum overnight, were then washed in water and incubated in goat anti-human IgG, IgA, and IgM for 2 h at room temperature; they were then washed and incubated in ^{125}I-labeled rabbit antiserum to goat globulin for 2 h. The beads were washed again, placed in tubes, and counted in a Packard Auto-Gamma counter.

The counts obtained with the herpes antigen and the control antigen were plotted on semilogarithmic graph paper, and a dose-response curve was drawn. Specific counts for both total HSV antibody and class-specific HSV antibody were determined by subtracting counts obtained with the control antigen from counts obtained with the viral antigen incubated with the same serum. Comparative calculations of endpoints by the use of specific counts, twofold difference in counts, and extrapolation of antigen curve to control curve indicated the use of specific counts to be the best method of determining endpoints (J. J. Ratner, Ph.D. thesis, University of Texas Health Science Center, San Antonio, 1978). The endpoint was that dilution of serum which showed a 100-specific-count difference between the antigen and control values. The titer is expressed as the reciprocal of the endpoint.

Treatment of specimens with protein A. Protein A, found in the cell walls of certain strains of *Staphylococcus aureus,* has receptors for the Fc portion of the IgG molecule (5, 10). Treatment of the serum specimens with protein A removed most of the IgG present (see below).

To prepare protein A, 10 liters of tryptic soy broth was inoculated with a 24-h broth culture of *S. aureus* Cowan strain 1 (kindly supplied by Ciro Sumaya, Department of Pediatrics, University of Texas Health Science Center at San Antonio) and incubated for 48 h at 37°C with mild agitation (10). Bacteria were pelleted by centrifugation at 2,500 × *g* for 20 min. The supernatant fluid was discarded; the bacteria were washed once in PBS, suspended in 3% Formalin in PBS, and incubated for 30 min at room temperature. The bacteria were then washed three times in PBS, diluted to 50% (vol/vol) with PBS containing 0.05% sodium azide, and stored at 4°C. Immediately before use, the bacterial suspension was washed and suspended to 50% with PBS; this preparation is hereafter called protein A.

Serum (0.4 ml) was added to 3.6 ml of protein A or PBS and incubated at 37°C for 10 min (10). The mixture of serum and protein A was centrifuged at 2,500 × *g* for 20 min; the supernatant fluid was removed, and the volume of the absorbed sera was adjusted to that of the control so that the final dilution was 1:10.

RID. The amounts of IgG, IgA, and IgM were quantitated in sera, before and after absorption with protein A, by using commercial radial immunodiffusion (RID) plates (Kallestad Laboratories, Chaska, Minn., and Behring Diagnostics, Somerville, N.J.).

Virus neutralization test system (plaque reduction). Protein A-treated and control sera were heat inactivated at 56°C for 30 min. Fresh-frozen guinea pig serum, used as a source of complement, was added to the medium at a final dilution of 1:30. This complement-medium mixture was calculated to contain 36 to 48 50% hemolytic complement units per ml (1) and was used as diluent in the preparation of virus dilutions and further serum dilutions.

HSV-1 virus dilution (0.3 ml; previously calculated to produce approximately 100 plaques) was added to 0.3 ml of each serum dilution and incubated for 1 h at room temperature. The virus control consisted of equal parts of diluent and the working dilution of virus suspension. After removal of growth medium, 0.2 ml of each serum-virus mixture or diluent-virus mixture was added to duplicate human fetal lung cultures grown in 35-mm tissue culture plates. These plates were incubated and shaken at room temperature for 1 h. The inoculum was then removed; 1 ml of medium was added to the plates, which were then incubated at room temperature for 1 h. The medium was then removed, and 2 ml of 1% Immune Serum Globulin (Abbott Laboratories, North Chicago, Ill.) in medium was added to each plate. Plates were incubated for 40 to 48 h at 37°C. Culture medium was discarded and plaques were stained with a 1:10 dilution of 1% crystal violet solution in water. Plaques were counted, and the percent reduction was calculated by the following formula: percent plaque reduction = 100 − (average number of plaques in serum dilution/average number of plaques in virus control) × 100. The results were graphed, and the endpoint was determined as that dilution of serum which reduced the number of plaques by 50%. The titer is expressed as the reciprocal of the endpoint.

RESULTS

Concentrations of immunoglobulins in sera untreated and treated with protein A. The total amounts of IgG, IgA, and IgM present in sera before and after absorption with protein A were determined by RID. There was a 7- to 30-fold reduction in the amount of total IgG, a 1.1- to 3.8-fold reduction in IgA, and a 0- to 2.7-fold reduction in IgM (Table 1).

Comparison of HSV-1 class-specific serum antibody titers (by RIA) before and after protein A treatment. Treatment with

protein A (Table 2) removed most, and in two sera all, of the HSV-1 IgG; there was a reduction that ranged from 160- to >8,000-fold. Assuming that a twofold or greater change is significant, sera 1, 2, 5, and 7 showed substantial increases of HSV-1 IgA. Two sera (1 and 5) were unmasked by treatment with protein A. Although serum 4 showed no substantial change, HSV-1 IgA was determined only after protein A treatment. This unmasking was confirmed by repetition of the absorption and retesting for HSV-1 IgA. Again assuming that a twofold or greater change is significant, sera 2, 5, and 7 showed substantial reduction in HSV-1 IgM concentration.

TABLE 1. *Concentrations of immunoglobulins in sera untreated and treated with protein A, as determined by RID*

Serum no.	Immunoglobulin class	Concn (mg/100 ml)		Fold reduction
		Untreated	Treated	
1	G	900	60	15.0
	A	100	88	1.1
	M	140	100	1.4
2	G	1,080	80	13.5
	A	228	200	1.1
	M	204	120	1.7
3	G	1,220	40	30.5
	A	276	136	2.0
	M	420	184	2.3
4	G	1,040	60	17.3
	A	200	120	1.7
	M	140	140	0.0
5	G	1,140	160	7.1
	A	76	20	3.8
	M	49	<20	>2.5
6	G	1,240	160	7.8
	A	160	120	1.3
	M	120	49	2.4
7	G	1,340	180	7.4
	A	200	120	1.7
	M	68	28	2.4
8	G	1,000	140	7.1
	A	260	120	2.2
	M	228	84	2.7

Comparison of total serum immunoglobulins (by RID) and HSV-1 class-specific antibodies (by RIA) before and after treatment with protein A. Table 3 summarizes the results of absorption studies showing the relationship between total immunoglobulins and HSV-1 class-specific antibodies in individual sera. For IgG and IgM there was a direct relationship between total immunoglobulin content and the titers against HSV-1; they both decreased. However, for IgA, there was an inverse relationship; the total IgA decreased while the titers of HSV-1 IgA increased. This indicates that the amount of HSV-1 IgA is only a small part of the total serum IgA and that its detection is dependent upon removal of IgG. These experiments showed that specific removal of IgG was necessary for more accurate detection of HSV-1 IgA.

Comparison of HSV-1 neutralizing antibody titers (by plaque reduction) before and after protein A treatment. More than 92 to 99% of the serum neutralizing activity was lost by removal of IgG due to treatment with protein A (Table 4).

DISCUSSION

Treatment of sera with protein A reduced the amount of total immunoglobulin levels for all three classes when determined by RID. These same sera that showed a decrease in all three immunoglobulin classes by RID showed, by RIA, a decrease for HSV-1 IgG (as expected) and, in addition, for HSV-1 IgM. Three of eight

TABLE 2. *Comparison of HSV-1 class-specific serum antibody titers (RIA) before and after protein A treatment*

Serum	Titer								
	IgG			IgA			IgM		
	Before	After	Fold change	Before	After	Fold change	Before	After	Fold change
1	180,000	70	2,571 (−)	<10	120	>12.0 (+)	16	<10	>1.6 (−)
2	80,000	<10	>8,000 (−)	22	45	2.0 (+)	68	<10	>6.8 (−)
3	68,000	50	1,360 (−)	45	50	1.1 (+)	<10	<10	0
4	60,000	<10	>6,000 (−)	<10	18	>1.8 (+)	<10	<10	0
5	25,000	50	500 (−)	<10	300	>30.0 (+)	82	22	3.7 (−)
6	16,400	90	182 (−)	14	10	1.4 (−)	15	<10	>1.5 (−)
7	16,400	50	328 (−)	26	120	4.6 (+)	25	<10	>2.5 (−)
8	8,000	50	160 (−)	58	38	1.5 (−)	<10	<10	0

TABLE 3. *Comparison of fold change in total serum immunoglobulins (RID) and HSV-1 class-specific antibodies (RIA) before and after treatment with protein A*

Serum no.	Fold change					
	IgG		IgA		IgM	
	RID (total)	RIA (HSV-1)	RID (total)	RIA (HSV-1)	RID (total)	RIA (HSV-1)
1	15.0 (−)	2,571 (−)	1.1 (−)	>12.0 (+)	1.4 (−)	1.6 (−)
2	13.5 (−)	>8,000 (−)	1.1 (−)	2.0 (+)	1.7 (−)	>6.8 (−)
3	30.5 (−)	1,360 (−)	2.0 (−)	1.1 (+)	2.3 (−)	0
4	17.3 (−)	>6,000 (−)	1.7 (−)	>1.8 (+)	0	0
5	7.1 (−)	500 (−)	3.8 (−)	>30.0 (+)	>4.1 (−)	3.7 (−)
6	7.8 (−)	182 (−)	1.3 (−)	1.4 (−)	2.4 (−)	>1.5 (−)
7	7.4 (−)	328 (−)	1.7 (−)	4.6 (+)	2.4 (−)	>2.5 (−)
8	7.1 (−)	160 (−)	2.2 (−)	1.5 (−)	2.7 (−)	0

TABLE 4. *Comparison of HSV-1 neutralizing antibody titers (plaque reduction) before and after protein A treatment*

Serum no.	Neutralization titer		
	Before	After	Reduction (%)
1	9,000	27	99.7
2	4,000	25	99.4
3	9,000	90	99.0
4	10,000	15	99.4
5	1,300	12	99.1
6	440	<10	>97.8
7	500	15	97.0
8	130	<10	>92.4

(38%) sera tested showed a substantial increase in HSV-1 IgA after treatment with protein A. Three of eight (38%) sera tested before treatment with protein A showed no HSV-1 IgA, but after treatment showed the presence of HSV-1 IgA. Had these sera been regarded as negative, an incorrect assessment of the absence of HSV-1 IgA would have been made. One explanation for the increase in the amount of HSV-1 IgA (in some cases, an unmasking) is that there is so much HSV-1 IgG or HSV-1 IgM present that all the sites on the solid-phase antigen may be covered, preventing the attachment and detection of HSV-1 IgA.

The disparity seen (Table 3) between fold changes for RID and RIA after protein A absorption may be explained as selective subclass removal. If the specificities of the two tests are different, then protein A may selectively remove certain subclasses not measured by one or the other test, making the results disparate.

To understand the roles of serum HSV-1 IgG, IgA, and IgM in the recurrence of oral herpes lesions, serum samples taken in timed relationship to these lesions need to be studied. Treating the sera with protein A would be essential in determining (by RIA) either the presence or more accurate concentrations of HSV-1 IgA.

In summary, the use of sera treated with protein A allowed detection, by RIA, of greater concentrations of HSV-1 IgA and showed that HSV-1 IgG was responsible for more than 92 to 99% of serum HSV-1 neutralizing activity.

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