Solid-Phase Radioimmunoassay of Serum Immunoglobulin A Antibodies to Respiratory Syncytial Virus and Adenovirus

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A solid-phase radioimmunoassay for detecting respiratory syncytial virus and adenovirus serum immunoglobulin A (IgA) antibodies was developed. An antigen consisting of purified adenovirus type 2 hexons or a crude lysate of respiratory syncytial virus-infected cells was first adsorbed onto polystyrene beads. The coated beads were then incubated with dilutions of serum, and IgA antibodies which attached to the solid-phase virus antigen were subsequently detected with ¹²⁵I-labeled anti-human alpha antibodies. The anti-human alpha antibodies used were isolated by immunosorbent chromatography from rabbit antiserum produced by immunization with IgA purified from serum of an IgA myeloma patient. A total of 46 serum specimens from 13 patients with respiratory syncytial virus infections and 10 patients with adenovirus infections were tested. Complement fixation, homologous IgG and IgM radioimmunoassay, and heterologous IgA radioimmunoassay testing were also done. Specific values higher than 10,000 cpm were often reached with convalescent serum specimens, and positive-to-negative serum binding ratios of 50 or more were frequently obtained with lower serum dilutions. IgA titers of convalescent sera were from 1,000 to 16,000, and with few exceptions a fourfold or greater rise in the IgA titer was detected in the homologous IgA radioimmunoassay.

Highly sensitive solid-phase radioimmunoassay (RIA) procedures designed to detect immunoglobulin G (IgG) and IgM antibodies against rubella, herpes simplex, measles, and tick-borne encephalitis virus have recently been developed in our laboratories (1, 4, 7, 8, 12). The principle upon which these earlier assays are based was currently adapted for the detection of IgA-class serum antibodies against respiratory syncytial virus (RSV) and adenovirus. These two viruses were selected for the present study to analyze the humoral IgA immune response to both a membrane or enveloped virus and a naked icosahedral virus. Moreover, both viruses are known to cause respiratory infections, and it is anticipated that the basic IgA RIA methodology described here will later prove useful in the assay of viral antibodies in the nasopharyngeal secretions of patients with respiratory infections.

MATERIALS AND METHODS

Antigen preparations. Preparation of the RSV-infected cell lysate is described elsewhere in detail (16). Briefly, monolayer cultures of Vero cells were

infected at a low multiplicity with the Randal strain of RSV. When the cytopathic effect was 3 to 4+, the medium was replaced with 0.02 M sodium phosphate (pH 7.4) containing 0.14 M NaCl (PBS) and the cells were mechanically scraped off. The cells were then collected by low-speed centrifugation, resuspended in PBS, and homogenized for 1 min at 4°C in a Sorvall Omnimixer set at maximum speed. The pellet obtained after centrifuging the homogenate at 80,000 × *g* at 4°C for 60 min was resuspended in PBS by sonication at 4°C. The protein concentration was determined by the Lowry method (11) with bovine serum albumin as a standard; the concentration of the crude RSV antigen used in the present study was 4.3 mg/ml. It was stored at -20°C and upon thawing was again sonicated to obtain a homogeneous suspension.

The method used in the purification of the adenovirus type 2 hexon antigen is also reported elsewhere in detail (6). Briefly, adenovirus type 2 was grown in spinner cultures of KB cells (13). Virions and soluble antigens were obtained as previously described (10). The fraction containing the soluble antigens was separated on diethylaminoethyl-cellulose (14) and hexons were further purified by repeated crystallization. This method yielded a hexon preparation that was 95 to 98% pure, based on polyacrylamide gel electrophoresis with and without sodium dodecyl sulfate. The protein concentration of the hexon antigen preparation used in the present study was 10.5 mg/ml. The antigen was stored at -60°C until used.

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Serum specimens. Acute- and convalescent-phase serum specimens from patients with acute respiratory infections were selected on the basis of a significant increase in complement fixation titers against RSV or adenovirus. The sera were obtained from the virus diagnostic laboratories in Turku and Uppsala and from the Virus Department, National Bacteriological Laboratory, Stockholm (through the courtesy of Monica Grandien). A total of 13 serum pairs from patients with RSV infections and 10 pairs from patients with adenovirus infections were included. The clinical history, including the day of onset of the disease, was available from only a few of the patients.

Purification of human IgA, IgG, and IgM. Human IgA purified from the sera of two IgA myeloma patients was used for the production of rabbit anti-human alpha antibodies and for the preparation of IgA immunosorbent columns. Myeloma serum was diluted to 20 mg of IgA per ml and then subjected to salt precipitation with ammonium sulfate at 4°C. The precipitate obtained at 0.40 saturation was collected by centrifugation, redissolved by dialysis against 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 8), and further purified by chromatography on diethylaminoethyl-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with the same buffer. After the column was washed with 1 bed volume of starting buffer, bound protein was eluted with a gradient of from 0 to 0.7 M NaCl in 0.1 M Tris-hydrochloride (pH 8). IgA-containing fractions, as determined by immunodiffusion, were pooled and concentrated by negative pressure dialysis. The concentrate (50 to 100 mg of protein in about 5 ml of volume) was then chromatographed on Sephadex G-200 (Pharmacia Fine Chemicals) equilibrated with 0.1 M Tris-hydrochloride (pH 7.7) containing 2 mM sodium ethylenediaminetetraacetic acid, 0.2 M NaCl, and 0.02% Na₃. IgA-containing fractions were pooled, concentrated, and stored at -30°C until use.

Normal human IgG was purified from Cohn fraction II of pooled sera (kindly supplied by KABI AB, Stockholm) by ion exchange chromatography and gel filtration as described above. Human IgM was isolated from the serum of a patient with macroglobulinemia and was purified by ion exchange chromatography and gel filtration as described above.

Antisera. Antisera to human IgA were prepared in New Zealand White rabbits (purchased from Försvarsets Fabriksverk, Åkers Styckebruk, Sweden). Antigen (1 mg/ml) was emulsified with an equal volume of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.), and each animal received an intra-muscular injection of 0.5 ml in each hind leg on days 0, 14, and 28. Two weeks after the third injection, a serum sample was taken, and the antibody titer was determined. Rabbits with high titers were immediately bled and, after 3 weeks, boosted with antigen. Sera were kept frozen at -30°C until use.

Porcine antisera specific for the mu chain of human IgM and the gamma chain of human IgG were obtained from Orion Diagnostica (Helsinki, Finland).

Isolation of specific antibody. Rabbit antiserum to human IgA was allowed to react successively with human IgG and human IgM coupled to CNBr-activated (15) Sepharose-6BCL (Pharmacia Fine Chemi-

cals, Uppsala, Sweden) to remove contaminating antibodies. The eluate was then passed through a column packed with Sepharose-conjugated human IgA. After washing with 10 volumes of Tris-buffered salt (pH 7.7) and 2 volumes of 0.5 M NaCl, specifically bound antibody was desorbed by elution with 0.1 M glycine-hydrochloride-0.5 M NaCl (pH 3). The eluate was dialyzed against 0.1 M Tris-hydrochloride (pH 8), concentrated to 5 to 10 mg of protein per ml, and further purified on Ultrogel AcA-34 (LKB-Produkter AB, Stockholm). Fractions containing monomeric antibody were pooled, concentrated to a final concentration of 5 to 10 mg/ml, and kept frozen at -30°C until use.

Antibodies specific for the human mu and gamma chains were isolated by immunosorbent chromatography on AH-Sepharose (Pharmacia Fine Chemicals) to which purified human IgM and IgG, respectively, had been coupled by a glutaraldehyde bridge (3). Bound antibodies were desorbed by elution with 3 M NaSCN, dialyzed immediately against PBS, concentrated, and stored at -60°C until use.

Iodination. Iodination of the purified anti-human alpha, mu, and gamma antibodies was done with a chloramine T procedure (5). The iodination reaction was stopped, and separation of the iodinated antibodies from unincorporated iodine was done by chromatography on Sephadex G-25 Fine (Pharmacia Fine Chemicals) equilibrated with PBS containing 0.5% bovine serum albumin and pre-eluted with PBS. Specific activities of 5 to 20 $\mu\text{Ci}/\mu\text{g}$ were obtained, and the iodinated antibodies remained active for at least 8 weeks when stored at 4°C in PBS containing 20% glycerol, 1% bovine serum albumin and 0.1% Na₃. The specificity of each of the three iodinated indicator antibodies was suggested by RIA analysis of fractions obtained by chromatography of an RSV convalescent serum on BioGel, A5m, 200 to 400 mesh (BioRad, Richmond, Calif.) equilibrated in PBS containing 0.5% Tween 20 and 0.1% Na₃. The immunoglobulin class specificity was further confirmed by identical immunoglobulin class-specific rubella RIA using IgM-negative but IgG- and IgA-positive, and IgG-positive but IgM- and IgA-negative serum specimens.

RIA procedure. The viral antigens were diluted in PBS and adsorbed on 6.4-mm-diameter polystyrene beads (Precision Plastic Ball Co., Chicago, Ill.) at a concentration of 5 to 7 μg per bead. After an overnight incubation at room temperature, unadsorbed antigen was removed and the beads were dried in a room-temperature air flow. Serum dilutions were made in PBS containing 20% heat-inactivated pig serum, 2% Tween 20, and 0.1% Na₃. Samples (200 μl) were added to disposable plastic tubes, and an antigen-coated bead was added to each. After 1 h at 37°C, unbound immunoglobulins were aspirated, and the beads were washed twice with 5 ml of tap water. ¹²⁵I-labeled anti-human alpha, mu, or gamma antibodies were diluted in Eagle minimum essential medium containing 10% heat-inactivated calf serum, 1% Tween 20, 0.5% lactalbumin hydrolysate, and 0.1% Na₃, and one 200- μl aliquot was added to each tube. Incubation was at 37°C for 1 h with the IgA or IgG RIA and 16 h with the IgM RIA. Unbound iodinated antibodies were then removed, and the beads were again washed. The

beads were finally transferred to clean tubes, and bound radioactivity was assayed.

The assays were standardized in two ways. First, the absolute amount of the iodinated anti-human alpha antibodies used per tube was that which gave 10,000 cpm bound when incubated for 1 h at 37°C with a bead coated with 2 µg of purified human IgA. This was obtained by diluting the label to 150,000 to 200,000 cpm/200 µl. The amounts of the iodinated anti-human mu (100,000 to 130,000 cpm/200 µl) and gamma (40,000 to 60,000 cpm/200 µl) antibodies used per tube were similarly standardized to give 2,500 cpm bound to human IgM- and IgG-coated beads, respectively. Second, interassay variation was compensated for by including positive and negative reference sera in each assay.

The RIA results were expressed as serum titers. In calculating the serum IgA endpoint titers, the cut-off point used was three times the cpm value of the negative control serum, with the proviso that the cut-off value be at least 500 in the RSV assay and 250 in the adenovirus assay. Before making endpoint titer calculations, appropriate buffer blank cpm corrections were always made. In the event that a test serum had a shallow dilution-versus-cpm curve, only the linearly declining part of the curve or its extension was used. Endpoint titers in the IgM and IgG RIAs were calculated in a similar fashion, except that a minimum value of 150 was used for the cut-off line.

RESULTS

Representative cpm-versus-serum dilution curves obtained with the RSV IgA RIA are shown in Fig. 1. Similar representative data for the adenovirus IgA RIA are shown in Fig. 2. The buffer blank cpm values for both assays were very low, usually only 70 to 150 cpm in the RSV assay and 50 to 70 cpm in the adenovirus assay. Negative control sera also gave low cpm values in both assays (data not shown). In the RSV IgA RIA, nonspecific IgA binding resulted in cpm values of only 100 to 200 at the starting serum dilution of 1:62.5. In the adenovirus assay, the nonspecific IgA binding was even lower. The higher buffer blank and nonspecific IgA binding values in the RSV IgA RIA may be related to the use of a membrane type of antigen compared to the use of a purified protein antigen in the adenovirus assay. Convalescent serum cpm binding in both the RSV and the adenovirus IgA RIAs was very high (Fig. 1 and 2). The calculated positive-to-negative serum cpm binding ratios were often greater than 50.

The RSV IgA RIA titers of acute and convalescent specimens from 13 patients with RSV infections are presented in Table 1. RSV IgG RIA, IgM RIA, and complement fixation titers as well as adenovirus IgA RIA titers are also given. The RSV IgA titers of convalescent specimens were from 1,000 to 16,000. Two patients (P.S. and T.L.) were found to have a twofold

increase in the RSV IgA titer, while the remaining patients had a fourfold or greater increase. All 13 patients had a fourfold or greater increase in RSV IgG RIA titers. RSV IgM antibodies

FIG. 1. Representative results of the RSV IgA RIA with acute- and convalescent-phase serum specimens from two patients with RSV infection.

FIG. 2. Representative results of the adenovirus IgA RIA with acute- and convalescent-phase serum specimens from two patients with adenovirus infection.

TABLE 1. *RSV and adenovirus antibody titers in acute- and convalescent-phase serum specimens of patients with RSV infection*

Patient	Age (yrs)	Time after onset (days)	RSV antibody titer by:				Adenovirus antibody titer by RIA IgA
			CF ^a	RIA IgM	RIA IgG	RIA IgA	
T.S.	3	10	4	<62.5	250	2,000	500
		20	128	500	4,000	8,000	500
A.S.	2	3	<4	<62.5	<62.5	125	250
		13	8	4,000	500	4,000	250
H.V.	78	8	8	<62.5	4,000	1,000	250
		18	32	2,000	32,000	16,000	250
R.K.E.	67	Acute	10	<62.5	4,000	1,000	250
		Convalescent	40	250	8,000	4,000	500
A.G.	2	Acute	<5	<62.5	NT ^b	1,000	4,000
		Convalescent	40	1,000	NT	8,000	4,000
M.A.S.	5	Acute	<5	<62.5	2,000	250	250
		+ 80 days	80	2,000	16,000	8,000	2,000
P.S.	2	Acute	<5	<62.5	1,000	500	<62.5
		Convalescent	80	<62.5	4,000	1,000	<62.5
T.L.	88	Acute	5	500	4,000	2,000	4,000
		Convalescent	40	<62.5	16,000	4,000	4,000
S.L.	79	Acute	5	<62.5	2,000	1,000	500
		+ 11 days	80	1,000	64,000	4,000	500
A.F.D.	77	Acute	<5	125	1,000	1,000	1,000
		Convalescent	40	250	8,000	4,000	1,000
A.K.L.	12	Acute	<5	<62.5	2,000	250	250
		Convalescent	40	<62.5	8,000	2,000	250
I.A.	50	Acute	<5	<62.5	2,000	1,000	250
		Convalescent	80	1,000	32,000	16,000	250
S.N.	80	Acute	<5	500	4,000	1,000	1,000
		+ 20 days	40	8,000	64,000	8,000	500

^a CF, Complement fixation.^b NT, Not tested.

were detected in at least one of the two serum specimens obtained from 11 of the 13 patients, with 10 of the 11 pairs having a fourfold or greater increase in IgM titers.

The adenovirus IgA RIA titers of paired serum specimens from 10 patients with adenovirus infections are presented in Table 2. As with RSV convalescent sera, the virus-specific IgA RIA titers of adenovirus convalescent sera ranged from 1,000 to 16,000. The IgA titers of paired serum specimens did not increase in one patient (P.S.), increased twofold in one patient (F.B.), and increased fourfold or more in the remaining eight patients. Adenovirus IgG RIA titer increases were similar to the adenovirus IgA RIA titer increases.

A significant increase in the heterologous virus IgA RIA titer was found in the paired specimens of only 1 of the 23 patients studied (Table 1, patient M.A.S.). This patient was a five-year-old girl who had been hospitalized for several weeks and who apparently experienced an adenovirus infection subsequent to contracting an RSV infection. Significant increases in the adenovirus IgG RIA, IgM RIA, and complement fixation

titers were also found in this patient's paired serum specimens.

DISCUSSION

The data presented in this report demonstrate that the described RIA method has the sensitivity and reliability needed for the detection of virus-specific serum IgA antibodies. Antigenic specificity of the assays was shown by the complete lack of cross-reactivity between the RSV and the adenovirus IgA antibody responses. No direct comparison can be made between the sensitivity levels of the IgA RIA and conventional serological techniques, because the complement fixation test does not measure IgA antibodies. Such a comparison can be made indirectly, however, since the IgA RIA titers were similar to the IgG RIA titers, which were 12.5 to 500 (usually 100) times higher than the complement fixation titers.

The RSV IgA titers of convalescent serum specimens were usually lower than the IgG titers, but were several times higher than the IgM titers (Table 1). In contrast, the adenovirus IgA titers of convalescent serum specimens were ap-

TABLE 2. Adenovirus and RSV antibody titers in acute- and convalescent-phase serum specimens of patients with adenovirus infection

Patient	Age (yrs)	Specimen	Adenovirus antibody titer by:				RSV antibody titer by RIA IgA
			CF ^a	RIA IgM	RIA IgG	RIA IgA	
P.S.	5	Acute	20	250	4,000	4,000	250
		+ 27 days	80	250	4,000	4,000	250
A.L.	30	Acute	5	125	500	500	1,000
		Convalescent	>160	16,000	32,000	16,000	1,000
C.P.	18	Acute	5	500	1,000	500	250
		Convalescent	160	4,000	16,000	4,000	250
P.H.	20	Acute	<5	125	1,000	250	500
		+ 7 days	80	500	16,000	2,000	500
H.N.	10	Acute	<5	125	250	125	125
		+ 12 days	40	2,000	16,000	1,000	125
F.B.	5	Acute	<5	250	2,000	2,000	1,000
		Convalescent	40	500	8,000	4,000	1,000
E.K.	19	Acute	<5	1,000	1,000	1,000	1,000
		+ 19 days	80	16,000	16,000	4,000	1,000
D.J.	21	Acute	10	125	4,000	1,000	250
		Convalescent	80	2,000	32,000	16,000	250
A.B.	3	Acute	<5	125	<62.5	<62.5	125
		+ 18 days	160	2,000	16,000	16,000	250
D.J.	3	Acute	<5	250	2,000	1,000	500
		+ 42 days	20	4,000	4,000	4,000	500

^a CF, Complement fixation.

proximately the same as the IgG and IgM titers (Table 2). Although the virus-specific IgA titers of convalescent serum from RSV- and adenovirus-infected patients were similar, not all viruses elicit such a strong serum IgA response. For example, with the RIA methodology described herein, it has recently been determined that rubella virus-specific IgA levels in convalescent serum specimens are approximately 10-fold lower than the comparable IgA responses in RSV or adenovirus infections (P. Halonen, unpublished data). Together, these results suggest that the class-specific humoral immune response elicited during a virus infection will generally conform to a pattern characteristic of that particular virus.

A prolonged persistence of both RSV and adenovirus serum IgA antibodies may occur, since RSV IgA antibodies were detected in each of the adenovirus-infected patients and, with one exception (a 2-year-old), adenovirus IgA antibodies were detected in each of the RSV-infected patients. In the present experiment, however, the actual picture of adenovirus IgA persistence may be complicated by the known existence of a large number of adenovirus serotypes. In fact, it was surprising just how well the highly purified adenovirus type 2 hexon antigen allowed the detection of the class-specific antibody responses in serum specimens from patients with adenovirus infections of unknown serotypes.

The number of days between the onset of the

disease and the collection of serum specimens was not available for the majority of the patients analyzed. For this reason, no final conclusion can be made concerning the diagnostic value of detecting virus-specific IgA antibodies in serum specimens by the described RIA methodology. The apparent persistence of serum IgA antibodies, however, would tend to rule against such an application. Therefore, the diagnostic potential of the IgA RIA appears to be in the assay of virus-specific IgA antibodies in secretions, particularly in nasopharyngeal secretions from patients with viral respiratory disease. Available studies of IgA antibodies in secretions have employed radioimmunoprecipitation techniques (2, 9). Such techniques, however, are more complex and less sensitive than the present IgA RIA, which suggests that the approach described here is particularly well suited for the detection of secretory IgA antibodies.

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