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Antigens of Pichinde Virus

I. Relationship of Soluble Antigens Derived from Infected BHK-21 Cells to the Structural Components of the Virion

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Antigens detected by the complement-fixation (CF) test were prepared from BHK-21 cells infected with Pichinde virus. The preparations contained two antigens demonstrable by immunodiffusion. The antigen present in abundance was heat stable, Pronase resistant, and had a molecular weight of 20,000 to 30,000 as estimated by gel filtration. Polyacrylamide gel electrophoresis of purified antigen demonstrated two low-molecular-weight polypeptides. An identical antigenic determinant was found by disrupting purified virus with Nonidet P-40; however, none of the viral polypeptides co-migrated with the polypeptides derived from purified CF antigen. Pronase digestion of disrupted virus did not alter antigenicity but degraded the viral peptides to sizes similar to those associated with the major CF antigen. These observations suggest that the major CF antigen of Pichinde virus is a cleavage product of the structural proteins of the virus.

Arenaviruses characteristically produce persistent infections in their natural hosts. The mechanisms responsible for the establishment and maintenance of these infections are poorly understood. Since persistence is most frequently associated with infections acquired in utero or neonatally, it was initially theorized that tolerance to the virus prevented an immune elimination of the virus (5). However, not only virus but viral antigens complexed to antibody have been detected in animals persistently infected with lymphocytic choriomeningitis (LCM) virus (19). Deposits of antigen-antibody complexes in the renal glomeruli have been demonstrated, and the deposits are thought to be of importance in the genesis of renal failure in persistently infected mice (20, 22). The apparent existence of tolerance, as manifested by virus persistence and an apparent absence of any measurable neutralizing antibody, and the presence of antigen-antibody complexes could be accounted for by tolerance to some viral antigens but not others. To examine this possibility, it is necessary to characterize the antigens induced by the virus and to establish their relationship to the known structural components of the virus.

Pichinde virus is a member of an arenavirus group that is relatively stable (16) and does not appear to be hazardous for laboratory personnel (4). The virus persistently infects the cricetine rodent, *Oryzomys albigularis*. Limited studies of the persistent infection in these rodents have demonstrated features similar to those previously reported for persistent LCM virus infection in mice (30).

In the present study, the antigens induced by Pichinde virus in BHK-21 cell cultures were characterized, and their relationship to the previously described virion polypeptides was examined. We found that the major soluble antigen induced in BHK-21 cell cultures infected with Pichinde virus was heat stable, of low molecular weight, and antigenically identical to an internal component of the virion.

MATERIALS AND METHODS

Cell culture. BHK-21 and Vero cell lines were grown in stationary or rolling monolayer cultures in plastic flasks (Corning). Cells were grown to confluency using Eagle minimal essential medium supplemented with 10 mM glutamine, 0.075% $NaHCO_3$, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid (HEPES) at pH 7.4, 100 U of penicillin per ml, 100 μg of streptomycin per ml and 10% heat-inactivated fetal calf serum. After infection, confluent monolayers were maintained on minimal essential medium as above but with 0.15% $NaHCO_3$ and 2% fetal calf serum.

Virus source and preparation. Pichinde virus, strain AN3730, was passaged three times in Vero cells and four to six times in BHK-21 cells after receipt (30). Virus stocks and antigens from infected cells were obtained by infecting monolayers of BHK-21 cells at a multiplicity of infection of 1 to 3 plaque-

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forming units per cell. After adsorption for 60 min at 37°C, the monolayers were washed once with phosphate-buffered saline (PBS) at pH 7.4, and approximately 60 to 80 ml of maintenance medium was added. The bottles of infected cells were then incubated at 37°C with rolling at 1.25 rpm for 48 to 72 h prior to harvesting virus and cells. Virus was harvested and purified as previously described (6, 8, 23). Cells were washed once in situ with borate-buffered saline (BBS) at pH 8.0, then scraped into cold BBS with a rubber policeman, and washed two more times by centrifugation. After the second wash, the cell pellet was resuspended in 20% (vol/vol) cold BBS for antigen extraction. Cell suspensions prepared in this manner were stored at −50°C prior to use. Virus assays were performed on monolayers of Vero cells in plastic tissue culture dishes (60 mm) as described elsewhere (16).

Isotopically labeled virus and cells were prepared utilizing 5.0 μCi of ^{3}H-labeled L-amino acid mixture per ml or 0.5 μCi of ^{14}C-labeled L-amino acid mixture per ml (New England Nuclear of Canada, Montreal). In some instances, 5 μCi of [^{3}H]glucosamine-HCl (New England Nuclear of Canada; specificity 5 to 15 Ci/mmol) per ml was used to label viral glycoproteins. Uridine-labeled virus was prepared as previously described (6). Twenty-four hours after infection, the cells were overlaid with maintenance medium containing the radiolabel, and the virus and cells were harvested 48 h later.

Soluble antigen extraction. The kinetics of appearance of complement-fixing (CF) antigen were examined, and maximum levels were detected 72 to 96 h after infection of cells with Pichinde virus (M. F. Carter, Ph.D. thesis, Baylor College of Medicine, Waco, Tex., 1972). Thus, cells harvested 72 to 96 h after infection were utilized as the source of antigen in the present study. Suspensions of infected cells prepared as described above were frozen and thawed twice to lyse the cells and then subjected to three 1-min cycles of sonic oscillation at maximum power (Bronwill Biosonik IV), with cooling on ice between cycles. The sonically treated lysates were then centrifuged at 12,000 × g for 30 min, and the pellet was discarded. The supernatant material was centrifuged at 100,000 × g for 60 min, and the pellet was again discarded. The material remaining in the supernatant contained the soluble antigens detectable by CF and immunodiffusion.

Antisera. Immune sera against Pichinde virus were raised in golden Syrian hamsters (LVG/Lak strain, Charles River/Lakeview) and in Hartley strain and random-bred guinea pigs (obtained locally). The animals were infected by intraperitoneal inoculation of 2.5 × 10^{4} plaque-forming units of stock virus diluted in Hanks balanced salt solution. The animals were bled by cardiac puncture at 4 and 5 weeks after infection, and the sera obtained from these bleedings were pooled and stored at −50°C in small portions. These sera will be referred to as Pichinde virus-immune hamster or guinea pig sera.

Antiserum to partially purified antigen was prepared in guinea pigs. Soluble antigen prepared as described above was centrifuged on a 5 to 20% sucrose gradient as subsequently described. The fractions from the gradient that contained the peak of CF activity were pooled and subjected to isoelectric focusing. The fraction from an isoelectric focusing gradient that contained the greatest antigen activity was dialyzed for 48 h against several changes of PBS and then emulsified in complete Freund adjuvant (Difco). A 0.5-ml portion of this material, which contained approximately 50 μg of protein, was inoculated into several sites in the thigh muscles of each of three guinea pigs. The animals were boosted with a second dose 14 days later. The animals were bled by cardiac puncture 10 and 20 days after the final inoculation.

Antisera to components of the virion were raised. A preparation of purified virus was divided into two portions (1:2), and the smaller portion was held on ice without further treatment. The larger portion was made 0.2% (vol/vol) Nonidet P-40 (NP-40; a gift of Shell Oil Co.), vortex mixed and incubated at 37°C for 45 min. The sample was then centrifuged for 2 h at 100,000 × g, and the resulting pellet and supernatant were collected. Portions of all samples were tested for infectivity. The untreated virus sample contained an excess of 10^{10} plaque-forming units per ml, whereas no infectious virus was detected in the pellet or the supernatant of the NP-40-treated sample. The three samples were emulsified in complete Freund adjuvant and used to inoculate three groups of eight guinea pigs each. At 3, 6, and 9 days after inoculation, two guinea pigs in each group were sacrificed, and blood, liver, and kidney samples were examined for infectious virus. Only those animals receiving untreated virus contained detectable amounts of infectious virus in the blood and organs. The remaining two animals in each group were bled at 21, 28, and 36 days after inoculation.

CF tests. CF tests for the detection of antigen or antibody were carried out in microtiter as previously described (31). A constant dilution (1:100) of heat-inactivated (56°C, 30 min), Pichinde virus-immune hamster serum was used for the detection of antigen. This dilution contained approximately 5 U of CF antibody. Antigen end points were corrected for dilution factors inherent in the test procedure, and the corrected values were used to calculate the number of antigen units per milligram of protein.

Immunodiffusion. Immunodiffusion was done on microscope slides (25 by 75 mm). The slides were precoated with 1.0 ml of a 0.5% aqueous solution of purified agarose (Sigma). Immediately prior to use, the slides were coated with 2.5 ml of molten 1% agarose dissolved in BBS with 0.1% NaN_3. Wells were punched with a template, and reagents were added to the wells. The slides were incubated for 72 h at room temperature in a humid chamber and washed for 5 to 7 days with daily changes of 0.15 M NaCl in the cold followed by a 2-h rinse in distilled water. The rinsed slides were then air-dried and stained with 0.25% Coomassie brilliant blue R-250 (Bio-Rad) in methanol-water-glacial acetic acid (45:45:10). The slides were destained in the same solvent without dye for 15 to 30 min as required to clear the background. Autoradiography on dried, stained ^{14}C-labeled immunodiffusion slides was done

by attaching the slides to a sheet of Kodak No-screen X-ray film and exposing them for 4 weeks.

Immunofluorescent staining. Indirect immunofluorescence tests of sera were performed using Vero or BHK-21 monolayers grown on round cover slips (18 mm). The monolayers were infected at a multiplicity of infection of 1 to 3 plaque-forming units per cell and used 24 to 48 h after infection. Cover slips were prepared for internal staining by thrice washing with PBS, air-drying, and then fixing the cells for 10 min in cold acetone. Cells were reacted with test serum for 30 min at 37°C, then washed three times, and reacted with fluorescein isothiocyanate conjugated goat antiserum to guinea pig immunoglobulin G (Cappel Laboratories) for an additional 30 min at 37°C. After the second incubation, the cover slips were washed twice with PBS and once with distilled water and then mounted in buffered glycerol (pH 8). The specificity of the reaction of antibodies to the partially purified CF antigen was tested by adsorption with disrupted virus. To 1 ml of a 1:5 dilution of serum was added 0.3 mg of virus that had been disrupted with 0.2% NP-40. After overnight incubation at 4°C, the serum was clarified by centrifugation at 100,000 × g for 60 min. The adsorbed serum was tested by indirect immunofluorescence along with unadsorbed serum, serum absorbed with intact virus, and serum adsorbed with NP-40 and diluent instead of virus.

Surface immunofluorescent staining of viable monolayers on cover slips was done by a previously described method (25). Briefly, monolayers of Vero cells were washed, reacted for 15 to 30 min with test serum at 20°C, washed free of test serum, and then fixed for 10 min with ice-cold phosphate-buffered formalin (pH 7.3) on ice. Residual formalin was washed off the cover slips; fluorescein isothiocyanate conjugated goat anti-guinea pig immunoglobulin G or rabbit anti-hamster immunoglobulin G was reacted with the cells for 30 min at 20°C. The cover slips were then washed and mounted as above.

Direct immunofluorescence staining of acetone-fixed cover slip monolayers was performed using a fluorescein isothiocyante conjugated globulin fraction from Pichinde virus-immune hamster serum. The conjugated globulin was reacted with the cells for 30 min at 37°C. The cover slips were then washed three times with PBS and mounted as above.

All immunofluorescent stains were examined using a Leitz Ortholux fluorescent microscope equipped with an HBO-200 mercury bulb, BG-38 and KP-490 excitation filters, and a K-530 barrier filter. Observations were made utilizing reflected incident light.

Gel filtration. Analytical gel filtration on Sephadex G-200 (Pharmacia) was carried out at room temperature in a upward flow column (1.5 by 55 cm) eluted with BBS (pH 8.0) containing 1:4,000 dilution of NaN_3. The hydrostatic head pressure was held constant at 11 cm of water, and the flow rate 12 ml/h. Void volume and total volume of the column were determined by chromatography of a mixture of 0.1% blue dextran (Pharmacia) and 0.04 mg of riboflavin per ml (Eastman). Marker proteins of known molecular weight (Schwarz/Mann) were utilized to calibrate the column for molecular weight estimation. Antigenic activity eluting from the column was determined by CF and by immunodiffusion.

Preparative gel filtration of material harvested from isoelectric focusing gradients was carried out using Bio-Gel P-100 or P-200 columns (Bio-Rad). A column (2.5 by 90 cm) was poured and equilibrated with BBS (pH 8.0). A 4- to 5-ml portion of pooled dialyzed material was made 10% in glycerol and applied to the column. Elution was at a rate of 12 ml/h. Antigenic activity was detected by CF and immunodiffusion.

Density gradient centrifugation. Rate zonal sedimentation was carried out in 5 to 20% (wt/vol) sucrose density gradients. Twelve-milliliter gradients were prepared in 0.1 M tris(hydroxymethyl)aminomethane buffer (pH 7.4) and formed with the aid of a gradient-making device. Centrifugation was for 20 to 22 h at 35,000 rpm (IEC rotor SB-283) at 4°C. Fractions were collected by bottom puncture, and the position of ^{125}I-labeled protein markers was determined with a gamma counter. Marker proteins were also located in the harvested fractions by immunodiffusion using specific antisera against the individual marker proteins. The sedimentation value for the antigenic activity was estimated relative to the marker proteins (15).

Preparative rate zonal sucrose gradients were used in purification of viral antigen. Twelve-milliliter 5 to 20% (wt/vol) sucrose gradients were made in cellulose nitrate tubes, and a 1.2-ml portion of the sample was layered on top of the gradient. The gradients were centrifuged for 22 h at 35,000 rpm at 4°C. Fractions were collected by bottom puncture, and the antigenicity was determined in each fraction by CF and immunodiffusion.

Isoelectric focusing. Isoelectric focusing was done in a glass column (110 ml, Ampholine LKB) according to the instructions supplied by the manufacturer. Two percent Ampholyne carrier ampholyte (pH range, 3.5 to 10) was used in a 0 to 46% (wt/vol) sucrose gradient. Samples were prepared for isoelectric focusing by dialysis overnight against 100 vol of 1% glycine at 4°C. Typically, a 10- to 15-ml portion of dialyzed samples was used to replace an equal volume of water in preparation of the less dense sucrose solution. A gradient was formed using a mixing device (LKB) to mix the more dense (46% wt/vol) and less dense (0% wt/vol) sucrose solutions. Anode and cathode electrolyte solutions were 1% H_2SO_4 in 60% (wt/vol) sucrose and 2% ethylenediamine in water. Voltage was regulated at 300 V for the first 24 h and then increased to 500 V for an additional 48 h. The temperature was maintained at 5°C using a refrigerated circulator. At the termination of the electrofocusing run, the anode tube was plugged, and the gradient was pumped out at the rate of 80 ml/h with a peristaltic pump. Fractions of 2.5 ml were collected, and the pH was determined at 5°C.

PAGE. Polyacrylamide gel electrophoresis (PAGE) in gel columns was carried out as previously described (23) using the discontinuous buffer system originally described by Laemmli (11). For optimal separation of the virion polypeptides, 15% acrylamide gels containing 0.2% sodium dodecyl sulfate

(SDS) and 0.5 M urea were used. Gels were slices into 1- or 2-mm segments using a Gilson automatic gel fractionator, and the radiolabeled polypeptides were eluted from segments into 0.3 ml of solubilizer (10% Beckman BBS-3 in water). After 4 to 12 h at room temperature, 8 ml of scintillation fluid was added. The fluid consisted of toluene, Fluoralloy-TLA (Beckman), and 10% BBS-3. The fractions were counted in a liquid scintillation counter, and in gels where double labeling was employed, the counts per minute of the ^{3}H-labeled amino acid were corrected for spillover from the ^{14}C channel, and the corrected counts per minute were plotted.

Protein determination. Protein concentrations were estimated by the method of Lowry et al. (14) using bovine albumin as a standard. Where the direct performance of Lowry protein determination was impossible due to interfering components in the sample (i.e., electrofocusing ampholyte or NP-40), the protein was precipitated overnight with 15% trichloroacetic acid on ice, and the precipitated proteins were redissolved in 0.1 N NaOH or 1% SDS and used for protein determination.

RESULTS

Soluble antigen detectable in cells infected by Pichinde virus. Two virus-specific antigens were detectable by immunodiffusion in soluble extracts of BHK-21 cells infected by Pichinde virus. The two antigens were found to differ in their thermal lability as well as their resistance to the proteolytic action of Pronase. One antigen was relatively stable at 56°C, whereas the other antigen could no longer be detected by immunodiffusion after a 20-min incubation at 56°C. When the soluble lysates were digested with 25 μg of Pronase per ml for 60 min at 37°C, the heat-stable antigen was also found to resist proteolytic digestion, whereas the heat-labile antigen was no longer detectable after proteolytic digestion. The antigen unaffected by heat and Pronase digestion was called the major antigen, and the heat-labile, Pronase-sensitive antigen was called the minor antigen. Neither antigen was affected by digestion with 25 μg of RNase per ml for 60 min at 37°C. By autoradiography of dried immunodiffusion slides, both antigens were shown to be labeled with [^{14}C]-arginine.

Some biophysical properties of the major antigen. A lysate of cells infected with Pichinde virus was centrifuged at 100,000 $\times$ g for 60 min and then applied to a G-200 Sephadex column which was previously calibrated with a series of proteins of known molecular weight. The antigenic activity, as assessed by CF, was eluted from the column in a peak corresponding to a molecular weight of 2×10^4 (Fig. 1). By immunodiffusion, only the major antigen was found after gel filtration, and it was located in the peak of CF activity.

The sedimentation rate of antigenic activity was found to be 3.5S (Fig. 2). The major peak of CF activity was shown by immunodiffusion to contain both the major and minor antigens. The minor antigen detected by immunodiffusion was found predominately in the faster sedimenting half of the antigen-containing peak and overlapped the slower sedimenting major antigen. Antigen that had been heated at 56°C for 60 min showed a marked decrease in activity in the peak.

By isoelectric focusing, a peak of CF antigenic activity focused at pH 5.2 with a variable shoulder at higher pH values (Fig. 3). The identity of the antigen peak at pH 5.2 was shown by

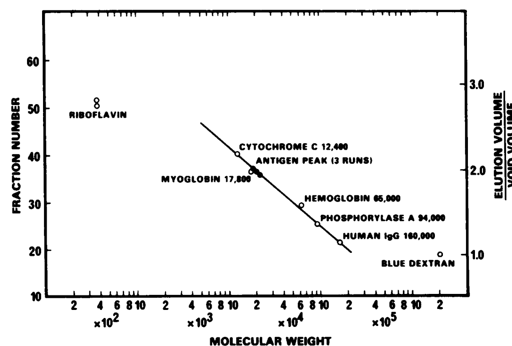

FIG. 1. *Estimation of the molecular weight of the CF antigen of Pichinde virus by gel filtration in G-200 Sephadex. A column (1.5 by 55 cm) was equilibrated with BBS (1:4,000 NaN_3) at pH 8 and standardized using the indicated marker proteins. A lysate of virus-infected BHK-21 cells was clarified by centrifugation at 100,000 $\times$ g for 60 min, and 1.2 ml of the supernatant was applied to the column. Fractions of 2 ml were collected and assayed for antigen by CF.*

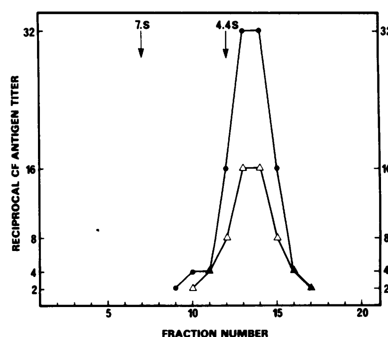

FIG. 2. *Sedimentation of soluble antigen in a 5 to 20% sucrose density gradient. A soluble extract of virus-infected cells was prepared and either heated to 56°C for 60 min (△) or used without further treatment (●). The sample was centrifuged for 22 h at 35,000 rpm (IEC SB-283 rotor). The antigenicity in collected fractions was determined by CF. The positions of human immunoglobulin G (7S) and hemoglobin (4.4S) marker proteins, run on a separate gradient, are shown.*

immunodiffusion to be the major antigen. The pattern of radiolabeled protein obtained when a parallel control cell lysate was focused on an identical gradient (pH 3.5 to 10) was similar to that obtained from infected cells. The soluble antigen would appear to comprise a relatively minor proportion of the total soluble protein of the infected cells.

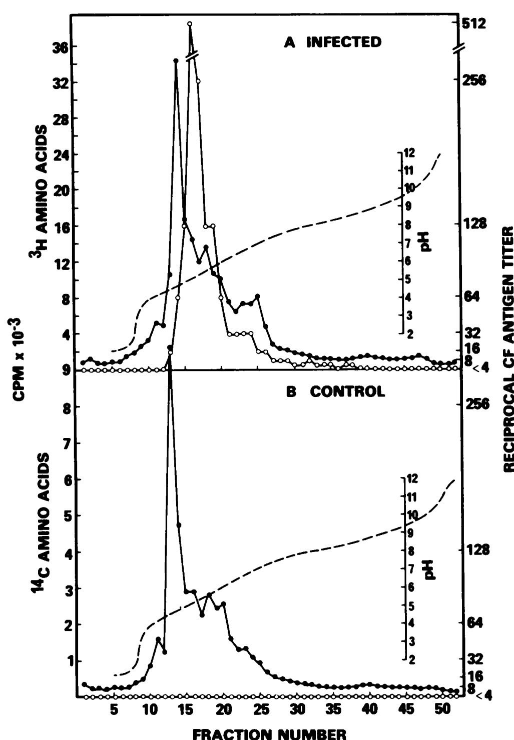

FIG. 3. *Isoelectric focusing of cell-derived Pichinde virus-specific and control antigens. Soluble lysates of infected (A) and control (B) BHK-21 cells were first centrifuged on preparative 5 to 20% sucrose gradients and then dialyzed against 1% glycine overnight. The dialyzed samples were incorporated into identical isoelectric focusing gradients (pH 3.5 to 10) and focused for 72 h at 300 V. Fractions were collected and assayed for radioactivity (●) and CF antigen activity (○). The broken line indicates pH.*

Taking advantage of the characteristics described above, a scheme to partially purify the major CF antigen from lysates of infected BHK-21 cells was devised. The methods used and the recovery of antigen at each step are shown in Table 1. In the experiment shown, 20-fold purification was achieved. Bio-Gel P-200 gel filtration of the antigen pool obtained by isoelectric focusing yielded a single peak of antigenic activity eluting from the column that was coincident with a peak of the ^{3}H-labeled amino acid label. Parallel preparations from noninfected cells yielded no peak of radiolabel eluting in the same region (Fig. 4). The approximate molecular weight of the eluting antigenicity relative to protein markers was 28,000.

Relationship of antigens derived from infected cells to components of Pichinde virus. The relationship between antigens extracted from infected cells and structural polypeptides of the virion was examined by fractionating the virus and examining the components for antigenic activity and polypeptide composition. The fractionation steps included disruption of the virus with 1% NP-40, separation of components of the disrupted virus by centrifugation in 20 to 50% sucrose gradients, treatment of the nonsolubilized component with RNase, and centrifugation on a 5 to 20% sucrose gradient of the RNase-treated material. A summary of the distribution of the structural polypeptides upon fractionation is shown in Table 2. Upon centrifugation of NP-40-disrupted virus in a 20 to 50% sucrose gradient, 72% of the radioactivity remained at the top of the gradient, and the remainder was found in a band with a density

TABLE 1. *Summary of purification of CF antigen from BHK-21 cells infected with Pichinde virus*

Stage of purification	Total protein recovered (mg)[a]	Total antigen recovered (CFU)[b]	Sp act (CFU/mg of protein)[c]	Purification index[d]
1. Crude 20% (vol/vol) cell suspension	100	>20,000	ND[e]	ND
2. 12,000 × g supernatant	50	15,000	300	1
3. 100,000 × g supernatant	24.5	14,745	602	2
4. 5 to 20% sucrose gradient antigen pool	11.0	14,080	1,280	4.2
5. Isoelectric focusing antigen peak (pH 5.2)	3.15	6,400	2,050	6.8
6. Bio-Gel P-100 antigen pool	0.51	3,060	6,000	20

[a] Protein content was determined by the method of Lowry et al. (14).
[b] CH_{50} antigen units were tested with 5 U of immune hamster serum. CFU, Complement-fixing unit.
[c] Ratio of total CF antigen units to total milligram of protein.
[d] Ratio of specific activity at indicated stage of purification to specific activity of 12,000 × g supernatant.
[e] ND, Not determined.

of about 1.24 g/ml. By using virus that had been doubly labeled with ^{3}H-labeled amino acids and [^{14}C]uridine, 46% and 53% of the ribonucleic acid was associated with the top gradient and the band at 1.24 g/ml, respectively. Both bands of disrupted virus contained antigenicity detectable by CF and by immunodiffusion. Electrophoresis of the material at the top of the gradient on an SDS-polyacrylamide gel revealed a polypeptide pattern similar to intact virus (Fig. 5A). The dense material appeared to contain enriched amounts of V_{II} and V_{IV} polypeptides and decreased amounts of V_I and no V_{III} (Fig. 5B).

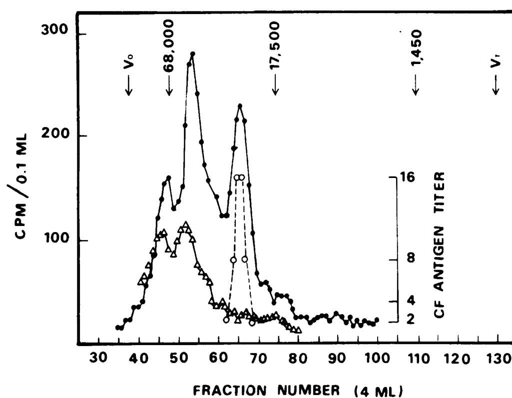

FIG. 4. *Bio-Gel P-200 gel filtration of Pichinde virus-specific CF antigen. Soluble lysates derived from virus-infected BHK-21 cells grown in the presence of 5 μCi of ^{3}H-labeled amino acids per ml and from control cells grown in the presence of 0.5 μCi of ^{14}C-labeled amino acids per ml were partially purified by rate zonal sedimentation and isoelectric focusing. The fractions containing the major peak of CF antigen and the corresponding fractions from the control preparation were dialyzed against BBS (pH 8) and then 4 ml of the dialyzed sample was applied to a Bio-Gel P-200 column (2.5 by 90 cm) equilibrated with BBS. The antigen-containing and control preparations were eluted in two separate runs, and the radioactivity and CF antigen in each fraction were determined. Symbols: ●, counts per minute of ^{3}H-labeled amino acids in material containing antigen; △, counts per minute of ^{14}C-labeled amino acids in the control preparation; ○, CF antigen activity. No CF antigen activity was detectable in the control preparation.*

When the material that banded at 1.24 g/ml in the 20 to 50% sucrose gradient was digested with 100 μg of RNase per ml and then recentrifuged on a 5 to 20% sucrose gradient containing 0.1% NP-40, a portion of the radiolabeled protein was rendered soluble. This soluble material sedimented at approximately 4.5*S* and contained all of the CF antigen activity previously associated with the material that banded at 1.24 g/ml. Analysis by immunodiffusion of the 4.5*S* fraction revealed the presence of precipitin bands that were identified with the antigens found in lysates of infected cells (Fig. 6). The 4.5*S* peak was shown by SDS-PAGE to contain a polypeptide migrating in the region of V_{II}, a polypeptide migrating near the V_{IV} polypeptide, as well as a reduced amount of the V_I polypeptide (Fig. 7). The material that sedimented through the 5 to 20% sucrose gradient only contained the V_I polypeptide. The apparent increase in V_{II} upon fractionation (i.e., 10.4% of purified virus to 14% in the starting material in the 4.5*S* fraction) is thought to reflect the underestimation of V_{II} in the presence of large amounts of V_I in the virion preparation.

Comparison of the polypeptide composition of purified major antigen derived from infected cells with the virion polypeptides. The major antigen, which had been labeled with ^{3}H-labeled amino acids, was purified as described above (Table 1), concentrated by trichloroacetic acid precipitation, and compared with the viral polypeptides by SDS-PAGE. Co-electrophoresis of the ^{3}H-labeled amino acid-labeled antigen

TABLE 2. *Relative distribution of the structural polypeptides of Pichinde virus after NP-40 disruption and RNase digestion*

Treatment of purified virus	Starting radioactivity (%)	Relative % of radioactivity in viral polypeptides[a]			
		V_I	V_{II}	V_{III}	V_{IV}
None	100	71	10.4	13.8	4.8
NP-40 disruption					
Soluble material	72	70 (50.4)[b]	8.4 (6.11)	16.7 (12.0)	5.3 (3.8)
Dense material (1.24 g/ml)	28	46 (12.8)	39.5 (11.1)	0 (0)	14.4 (4.0)
RNase digestion					
Dense material					
4.1*S* fraction	23.8%	16 (3.8)	60 (14.0)	0 (0)	24 (5.6)
Pellet	4.2%	92 (3.9)	8 (0.3)	0 (0)	0 (0)

[a] The relative percentage of radioactivity in individual polypeptides was estimated by dividing the counts per minute in each peak obtained by PAGE by the total counts per minute in V_I, V_{II}, V_{III}, and V_{IV}.

[b] Estimate of the total radioactivity of the starting material present in the polypeptide.

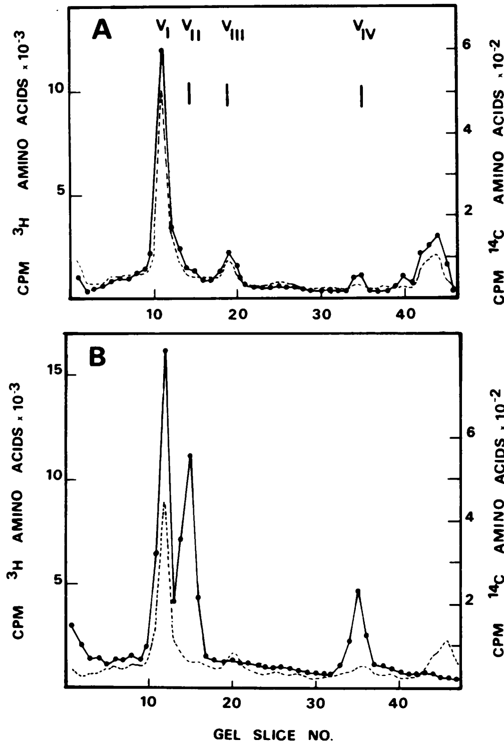

FIG. 5. *Polypeptide composition of the components obtained from Pichinde virus after NP-40 disruption. Purified Pichinde virus, radiolabeled with ^{3}H-labeled amino acids, was disrupted with 1% NP-40, layered onto a 20 to 50% (wt/wt) sucrose gradient containing 0.1% NP-40, and centrifuged for 120 min at 35,000 rpm. The material remaining on top of the gradient (A) and the material pelleting through the gradient (B) were harvested, adjusted with 1% SDS, 0.1% 2-mercaptoethanol, and 0.5 M urea, and prepared for SDS-PAGE. ^{14}C-labeled amino acid-labeled Pichinde virus (1,500 cpm) was mixed with approximately 3,000 cpm of the sample, and the mixture was subjected to co-electrophoresis on a 15% polyacrylamide gel containing 0.2% SDS and 0.5 M urea. Symbols: ●, counts per minute of ^{3}H-labeled amino acids in the sample; -----, counts per minute of ^{14}C-labeled amino acids in virus marker.*

with ^{14}C-labeled amino acid-labeled material from control cells purified in parallel revealed two unique polypeptides in the antigen preparation (Fig. 8A). These polypeptides had estimated molecular weights of 15,000 and 20,000. Co-electrophoresis of the antigen with purified virus revealed that neither of the two polypeptides present in the antigen preparation comigrated exactly with any of the virion structural polypeptides (Fig. 8B).

One explanation for the antigenic cross-reactivity between components of the virus and the major CF antigen in the absence of polypeptides with similar migration properties is that either one or both of the low-molecular-weight polypeptides of the purified antigen arises through proteolytic cleavage of the larger polypeptides of the virion. To examine this possibility, purified virus which had been radiolabeled with ^{3}H-labeled amino acids was disrupted by incubation with 1% NP-40 and 50 μg of RNase per ml at 37°C for 15 min. After disruption, the virus was divided into two portions, and Pronase (40 μg/ml) was added to one portion while a similar volume of buffer was added to the second portion. Incubation was continued for 30 min at 37°C, after which the samples were prepared for SDS-PAGE and immediately subjected to electrophoresis on 15% polyacrylamide gels. Pronase digestion generated two polypeptides with approximate molecular weights of 18,500 and 12,000 relative to marker proteins run on a parallel gel (Fig. 9).

The effect of Pronase digestion on the antigenic activity associated with the virus was examined by disrupting purified virus and digesting the preparation with Pronase at concentrations of 40 and 100 μg/ml. A portion of soluble antigen extracted from infected BHK-21 cells and clarified by centrifugation at 100,000 $\times g$ was treated with NP-40 and RNase, and samples were digested with 40 and 100 μg of Pronase per ml in the same manner. The antigenic activity in each of these preparations was compared by immunodiffusion (Fig. 10). The major antigen was detectable in both the untreated and Pronase-digested samples prepared from both cell-derived antigen and from purified virus. These results suggest that the antigenic portion of one of the larger virion polypeptides was Pronase resistant.

Detection of antigens by immunofluorescence. To confirm the similarity of the major CF antigen and the antigens associated with the virion (24), the distribution of the antigens in infected cells was examined by direct immunofluorescence using antisera prepared from different antigen sources. Sera from two guinea pigs immunized with partially purified CF antigen had CF titers of 1:64 against virus-induced antigen. A pool of these sera gave a band that was identical to the major antigen by immunodiffusion. Sera from animals injected with the dense component of purified virus disrupted with NP-40 produced one band, by immunodiffusion, when reacted with lysates of infected cells; this band was identified with the band detected by antisera prepared against purified major CF antigen (24). By indirect immunofluorescence using BHK-21 or Vero cells infected with Pichinde virus and guinea pig antiserum to the major antigen at a dilution of 1:16, a granular cytoplasmic staining was observed in acetone-

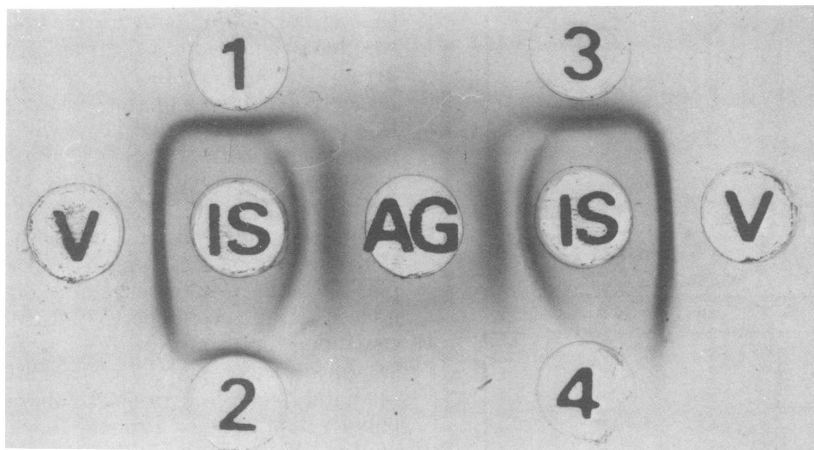

FIG. 6. *Immunodiffusion test of virion components obtained by NP-40 disruption and RNase digestion. Contents of the wells: (1) top band and (2) material that banded at >1.24 g/ml which we obtained from purified Pichinde virus by treatment with NP-40; (3) 4S material and (4) insoluble fractions obtained by digestion of material that banded at 1.24 g/ml with RNase; (IS) immune guinea pig serum; (AG) soluble antigen derived from infected cells; (V) purified virus disrupted with NP-40 and digested with RNase.*

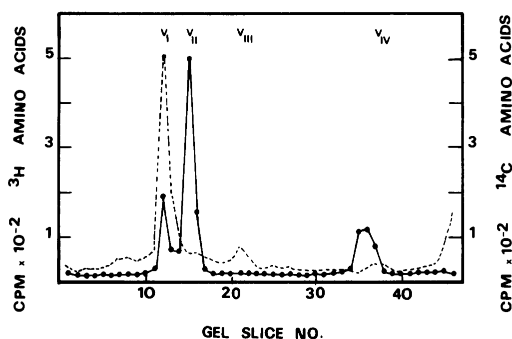

FIG. 7. *Polypeptides present in the soluble antigen liberated from material that banded at >1.24 g/ml by RNase treatment. The soluble, antigen-containing fraction from the dense material was prepared, and the gradient fraction containing the peak counts per minute and antigen was analyzed by SDS-PAGE. Symbols: ●, 3,000 cpm of sample radiolabeled with ^3H-labeled amino acids was subjected to co-electrophoresis with 1,500 cpm of ^{14}C-labeled amino acid-labeled Pichinde virus marker (-----).*

fixed cells (Fig. 11A). The staining pattern observed with antisera to the partially purified antigen was similar to that observed when the cells were reacted with immune sera from guinea pigs (Fig. 11C). Antisera against the insoluble component of the NP-40-disrupted virus produced a similar pattern (Fig. 11B). None of the antisera stained uninfected cells. Absorption of antiserum to the major antigen with virus disrupted by NP-40 abolished the cytoplasmic staining.

Indirect immunofluorescent staining of the surface of viable cells was performed using sera from hamsters or guinea pigs infected with the virus, antiserum against partially purified major antigen, and antiserum against the dense component of disrupted virus. Virus-specific staining of the surface of the infected cells was obtained with immune sera from both hamsters and guinea pigs but not with antiserum to the major CF antigen or with antiserum to the dense component of disrupted virus. In addition to demonstrating a similar cytoplasmic distribution of antigen detected by antibodies to major CF antigen and to antigens in the virion, these data suggested that the major CF antigen was not expressed on the surface of cells infected with Pichinde virus.

DISCUSSION

Antigens detectable by the CF test are produced in abundance by cells infected with arenaviruses (1, 2, 3, 9, 27, 32). Studies of the antigens induced by LCM virus demonstrated that soluble preparations with CF activity contained two antigens which were detected by immunodiffusion. These two antigens differed in their susceptibility to heat and to digestion with Pronase. One antigen with an estimated molecular weight of 43,000 was relatively resistant to heat and to digestion with Pronase. Present in lesser amounts was a second antigen that was digested by Pronase (2). In the present study, we have found by immunodiffusion two antigens in lysates of cells infected by Pichinde virus. The most abundant of the two antigens was found to be relatively stable upon heating. It was also resistant to the proteolytic action of Pronase. A molecular weight of 20,000 to 30,000 was esti-

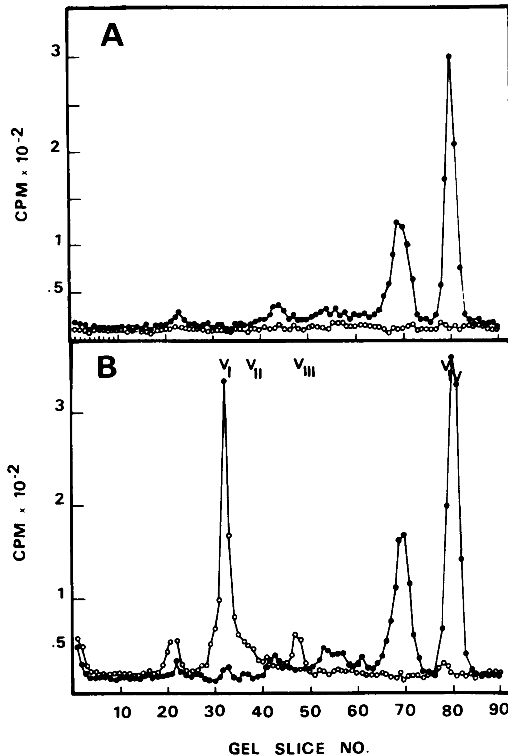

FIG. 8. *Polypeptide composition of purified major CF antigen. Major antigen, labeled with ³H-labeled amino acids, was purified by rate zonal centrifugation, isoelectric focusing, and Bio-Gel P-200 gel filtration. The purified antigen was precipitated with 15% trichloroacetic acid, then washed with 5% trichloroacetic acid and acetone, and dried. The dried protein was solubilized with 1% SDS and subjected to electrophoresis on a 15% polyacrylamide gel. (A) Comparison of antigen preparation with mock material. Symbols: ●, antigen purified from infected BHK-21 cells; ○, parallel purified ¹⁴C-labeled amino acid-labeled control material. (B) Comparison of the polypeptides in purified antigen with the virion structural polypeptides. Symbols: ●, purified antigen; ○, ¹⁴C-labeled amino acid-labeled virus marker.*

mated by gel filtration for this antigen; however, the antigen had a sedimentation coefficient of 3.5S, which closely agreed with the value reported for the antigen of LCM virus (2). By isoelectric focusing, the major antigen induced by Pichinde virus was found to have an isoelectric point of pH 5.2. The minor antigen induced by Pichinde virus was not present in sufficient concentrations in our soluble preparations to allow biophysical characterization.

When the major antigen was partially purified and injected into guinea pigs, antibodies that reacted by immunofluorescence with the cytoplasm but not with the surface of cells infected with Pichinde virus were raised. The granular pattern of intracytoplasmic staining observed by immunofluorescence was similar to that observed when immune sera were used. These observations suggest that the major antigen detected by CF accounted for the bulk of the antigen detected by immunofluorescence. The same conclusion has been reached in recent studies of LCM virus (25).

The results of early studies suggested that the CF antigens of LCM virus were not structural components of the virion. Infectious virus was readily sedimented by high-speed centrifugation, whereas the soluble antigens were not (28). Adsorption of antisera with soluble antigen preparations did not diminish their virus-neutralizing capacity (29). In addition, more recent studies have shown a discordant production of infectious virus and CF antigen both in L-cell cultures chronically infected with LCM virus (12) and in BHK-21 cell cultures infected

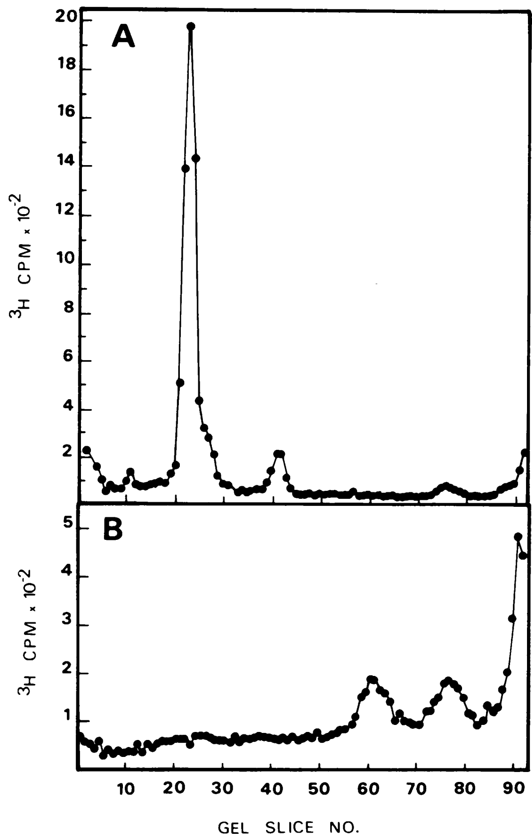

FIG. 9. *Effect of Pronase digestion on the virion polypeptides of Pichinde virus. A portion of purified Pichinde virus radiolabeled with ³H-labeled amino acids was disrupted by treatment with NP-40 and RNase. After disruption, the virus was divided into two portions and either left untreated (A) or digested with 40 μg of Pronase per ml for 30 min at 37°C (B). Both samples were analyzed by SDS-PAGE on 15% acrylamide gels.*

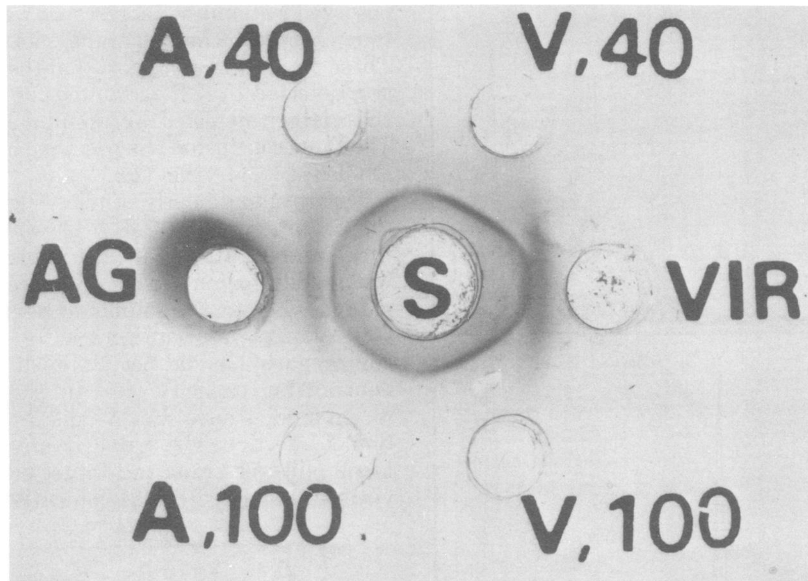

FIG. 10. *Identity of Pronase-resistant antigen from purified virus with the major antigen of infected cells. Purified virus (VIR) and soluble antigen (Ag) extracted from infected cells were pretreated with 1% NP-40 and RNase. Each sample was divided into three portions and either left untreated or digested with 40 or 100 μg of Pronase per ml. The center well contained guinea pig antiserum against Pichinde virus.*

by Pichinde virus and incubated in the presence of actinomycin D (23a).

In the present study, data which indicated that the internal components of the virion were antigenically identical to the major and minor antigens were obtained. Antisera raised from the dense material derived from the virus that had been disrupted with NP-40 reacted with the major antigen extracted from infected cell cultures. Gschwender and Lehmann-Grube (Abstr. Int. Congr. Virol., 3rd, Madrid, 1975) have recently reported similar data indicating that the CF antigen extracted from cells infected with LCM virus is located internally in the virion. The findings obtained in the present study with the Pichinde virus as well as those obtained with LCM virus support the concept that the CF antigen induced during the infection of arenavirus is an internal component of the virion.

Purified antigen derived from infected cells was found to contain two major polypeptides with estimated molecular weights of 15,000 and 20,000. Neither of these polypeptides co-migrated with any of the four structural polypeptides of the virion, although antigenic identity between the major CF antigen and components of disrupted virus was readily demonstrated by immunodiffusion. The antigenic determinant of the major CF antigen appears to reside in a Pronase-resistant portion of the molecule. The digestion of disrupted virus with Pronase generated two low-molecular-weight polypeptides similar in size to those observed in preparations of purified CF antigen derived from infected cells, and the antigenicity of the major antigen was not lost with Pronase digestion. These observations suggest that the major CF antigen derived from infected cells represents a product of proteolytic breakdown of structural molecules of the virus.

The identity of the structural polypeptide containing the major antigenic determinant was not conclusively demonstrated. The association of the antigenic activity with the dense component of the virus that had been disrupted with NP-40 indicates that the antigen is not associated with V_{III}, since this glycopeptide is totally solubilized by NP-40. The dense component of disrupted virus could be partially solubilized by digestion with RNase, and the solubilized material, which contained the antigenic activity, was enriched in V_{II} and V_{IV}. However, this material also contained appreciable amounts of V_I.

Little can be said about the possible function of the antigenic proteins in the virus. By virtue of their demonstrated presence within the virion and apparent association with the viral ribonucleic acid, they may function in a ribonucleoprotein complex as has been previously demonstrated for the soluble CF antigen of influenza virus (10, 13, 26). Since the arenaviruses lack a demonstrable core (7, 17), the

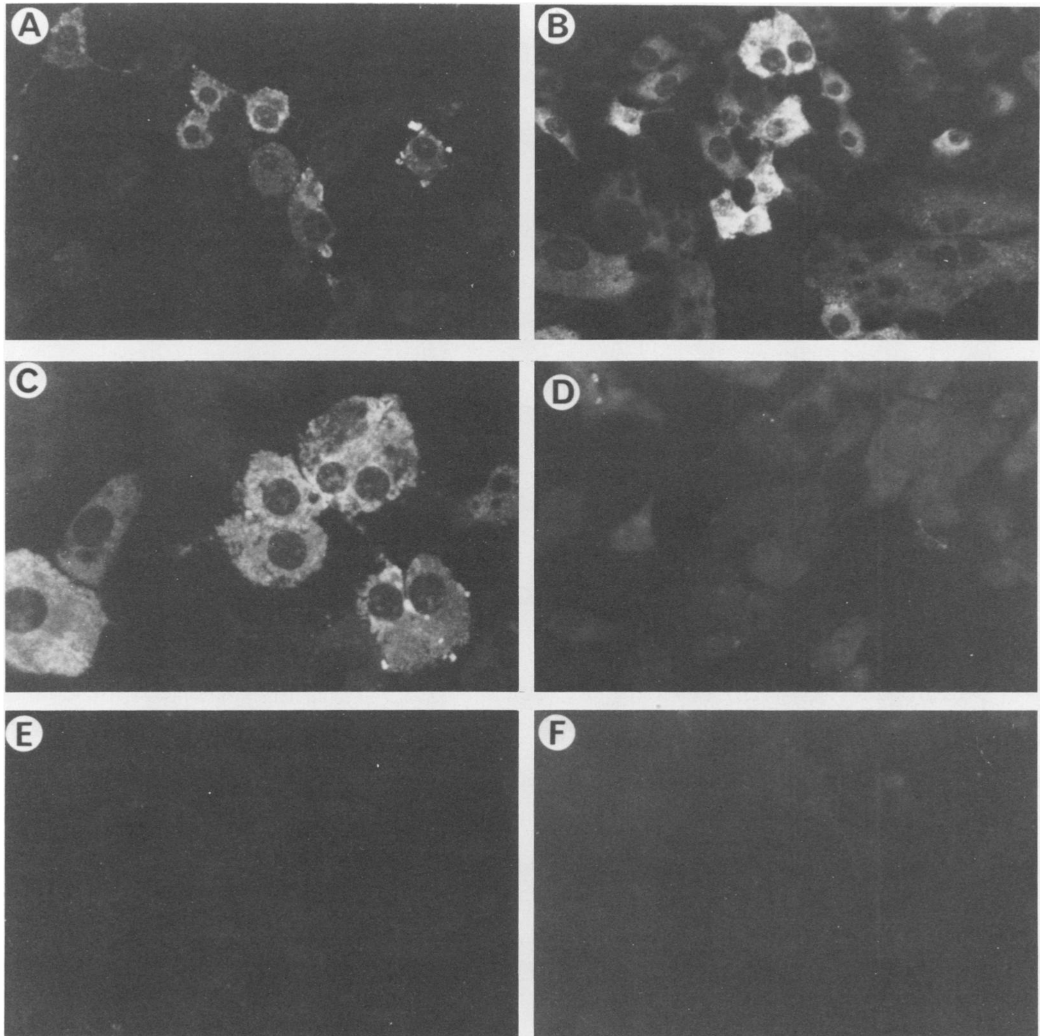

FIG. 11. *Indirect immunofluorescence stain of Pichinde virus-infected Vero cells by antiserum from guinea pigs injected with (A) partially purified, major CF antigen; (B) dense component of NP-40-disrupted virus, and (C) intact virus. Uninfected Vero cells reacted with the respective antisera and fluorescein isothiocyanate conjugated anti-guinea pig immunoglobulin G are shown in (D), (E), and (F).*

structural position occupied by such a ribonucleoprotein particle cannot be stated at this time.

The appearance of the CF antigen in the extracellular environment is of interest in regard to the host response to arenavirus infection, for it is in this location that the primary damage in the form of immune-complex disease is initiated (20, 21). CF antigens are synthesized with the production of minimal quantities of infectious virus in cells that have been chronically infected both in vitro (12) and in vivo (18). It is conceivable that animals infected with arenaviruses could mount an immune response to these CF antigens with relative unresponsiveness to the surface antigens of the virus. Because of the internal location of the CF antigens in the virion, antibodies to these antigens would not be expected to have neutralizing capabilities. The information available is compatible with the concept that in certain persistent infections, there may be an immune response to CF antigens, but the immune response to the antigens involved in virus neutralization may be deficient.

ACKNOWLEDGMENTS

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