Etiology of the Stomatitis Pneumoenteritis Complex in Nigerian Dwarf Goats

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ABSTRACT

The causative agent of stomatitis pneumoenteritis complex was isolated in domesticated goats and Vero cell culture. It was identified immunologically and morphologically as identical with the "Peste des Petits Ruminants" virus. There were cross reactions between stomatitis pneumoenteritis complex virus isolate and rinderpest virus by immunodiffusion and complement fixation tests but no cross neutralization. Goats recovered from stomatitis pneumoenteritis complex were protected against a challenge with rinderpest virus that was lethal to control goats. Ultrastructural morphology revealed intracytoplasmic and intranuclear inclusions made up of random arrays of fibrillar strands. Pleomorphic particles budded from the plasma membrane of infected cells and enveloped virions were seen extracellularly. Specific ferritin tagging was demonstrated in the stomatitis pneumoenteritis complex virus infected cells treated with homologous and peste des petits ruminants viral antibody systems but little, if any, tagging in the heterologous rinderpest system.

RÉSUMÉ

Les auteurs ont isolé l'agent causal du complexe: stomatite-pneumo-entérite, chez des chèvres domestiques et sur culture tissulaire Vero. Cet agent s'avéra identique au virus de la peste des petits ruminants, tant du point de vue immunologique que morphologique. Des épreuves de diffusion sur gélose et de déviation du complément donnèrent des réactions croisées entre le virus du complexe et celui de la peste bovine, mais pas de neutralisation réciproque. Les chèvres rétablies du complexe résistèrent à une infection expérimentale avec une souche du virus de la peste bovine qui entraîna la mort des chèvres témoins. L'utilisation de la microscopie électronique révéla la présence d'inclusions intra-cytoplasmiques et intra-nucléaires composées de rangées fortuites de cordons fibrillaires. Elle permit aussi de déceler, à l'extérieur des cellules, des particules pléomorphes bourgeonnées de la membrane plasmique de cellules infectées et de virions enveloppés. Les auteurs réussirent le marquage spécifique à la ferritine des cellules infectées par le virus du complexe et traitées avec des anticorps homologues ou de la peste des petits ruminants, contrairement à ce qui se produisit lorsqu'ils utilisèrent des anticorps du virus de la peste bovine.

INTRODUCTION

A disease named as Kata or stomatitis pneumoenteritis complex (SPC) had been described in dwarf goats in western and

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eastern Nigeria (5, 6, 10, 12). It proved to be transmissible and was characterized by stomatitis, pneumonia and enteritis that resulted in severe diarrhea. The similarities in clinical picture and pathological findings between this disease, rinderpert (RP) and Peste des Petits Ruminants (PPR) were reported (8, 9, 11). RP and PPR viruses are immunologically related as shown by cross protection and cross reaction in complement fixation tests (F. M. Hamdy, A. H. Dardiri and S. S. Breese, Jr. Abstr. Ann. Mtg. A. S. Microbiol, N.Y. p. 265. April 28-May 2, 1975).

Recently Nduaka and Ihemelandu (6) reported that the pneumoenteritis complex was previously designated by various names such as goat catarrhal pneumonia, pleuropneumonia and pneumoenteritis complex. More recently, Provost (7) proposed a multiple etiology of Kata disease and the possible implication of PPR virus, myoplasma and parainfluenza 3 virus.

The etiology of the stomatitis pneumoenteritis complex is not established and needs to be clarified because the disease has several implications. Economically, stomatitis pneumoenteritis complex is a source of great financial loss to farmers and stock owners in Nigeria and causes a serious meat deficiency for the Nigerian population. Workers have estimated that pneumonia, a pathological syndrome in the SPC, causes 80-90% mortality of infected goats; a loss of nearly $1,500,000 per year.

In an attempt to determine the etiology of the stomatitis pneumoenteritis complex syndrome, tissues from goats sacrificed during outbreaks at Abakaliki, Enugu and Nsukka of eastern Nigeria were requested to be examined at the Plum Island Animal Disease Center (PIADC). These tissues were supplied by the third author and the results of their examination are the subject of this report.

MATERIALS AND METHODS

Source of Inoculum

Tissue specimens from five goats that were involved during the outbreaks were collected and placed in suitable metal screw-capped glass containers. Vials were sealed, frozen with dry ice and shipped in CO_2 ice by air to the laboratory. At arrival they were still frozen and were stored at –70°C until examination.

Animals

Three adult goats with three to four week old nursing kids were initially used for testing the infectivity of the tissues from the Nigerian sacrificed goats (Table I). The three kids received an intramuscular inoculation of 5 ml of a specimen prepared from Nigerian goat tissues. The three adult goats were not inoculated and served as contact controls.

Three other goats (approximately one year old [Table I]) were inoculated intramuscularly with tissue suspension made from the first goat passage. The inoculum was pooled spleen and lymph node suspensions (10% wt/vol) from goats #3965 and #3968 (Table I). Each goat received 2 ml.

All the goats were kept indoors in environmentally controlled rooms. The animals were held under clinical observation until death or for nine weeks, and blood samples were collected at intervals for virus isolation and testing for antibody response.

A group of six adult goats were used to determine the infectivity of cell culture harvest of the virus isolated from blood of goat #3968 and quaternary passaged in Vero cells (Table III). Four goats were inoculated intravenously, and two were uninoculated and served as contact controls.

Inoculum

Ten percent (wt/vol) infected tissue suspension was prepared in phosphate buffer saline from a pool representing spleen, lymph node (LN) tissues, kidney and lung of the Nigerian goats. The suspension was centrifuged at 800 x g. To the largest part of the tissue suspension was added suitable concentration of antibiotics. The fluids harvested from tissue cultures showing cytopathic effect (CPE) were inoculated into suitable media to check for presence of bacteria, mycoplasma organisms and fungi. The inoculum was also tested in nine day old chicken embryos. Twelve eggs were inoculated on the chorioallantoic membrane with 50 µl of the prepared inoculum and examined daily for viability.

Reference Viruses

PPR virus was originally obtained from the Laboratoire National de l'Elevage et des Recherches Vétérinaires Dakar-Han (Sénégal) in 1968 through Dr. M. Rioche, Director. The virus was received as lyophilized lung extract and was resuspended in sterile distilled water and injected into susceptible goats, blood was collected at the peak of thermal response. The virus was then grown in bovine embryo spleen and Vero cell lines.

Attenuated and virulent forms of the Kabete "O" strains of RP virus were supplied by Dr. W. Plowright, East African Veterinary Research Organization, Muguga, Kenya as a lyophilized Vero cell culture passage. For this experiment, the virulent virus was passaged once in Vero cell culture.

PPR and Immune Goat Sera

The immunity of goats that recovered from infection or contact exposure with PPR virus was challenged with virulent PPR virus. Three weeks after challenge with 5 x 10^5 LD_{50}, blood was collected and serum was separated and stored at –70°C until used. RP sera likewise were prepared in goats inoculated with attenuated Kabete "O" strain RP followed by challenge with the virulent strain.

Vero Cell Culture

The Vero cell line culture was used for virus isolation, propagation and assay of neutralizing antibodies. The Vero cell line was obtained from the American Type Culture Collection. The medium used for growth and maintenance was LYE in Earle's balanced salt solution containing 0.05% lactalbumin hydrolysate, 0.005% yeast extract, 0.0015% phenol red and was supplemented with 5% fetal calf serum. One hundred units of penicillin and 100 μg of streptomycin per ml were incorporated. The final pH was 7.3.

Virus Isolation

Blood from inoculated and contact goats was obtained at time of fever, defibrinated and centrifuged at 800 x g, and the buffy coat cell (BCC) fraction collected from each specimen. Vero cell cultures were inoculated with 0.2 ml of the BCC preparation. After one hour incubation at 37°C to allow for virus adsorption or leukocyte seeding, the maintenance medium was added. The latter was changed every three days and the cultures were observed for development of CPE. Vero cell cultures were treated similarly and maintained as controls.

Complement Fixation (CF) Test

Antigens were prepared from LN samples from necropsied goats. A 10% (wt/vol) suspension was made in Veronal Buffer Diluent (3), subjected to three cycles of freezing and thawing and clarified by centrifugation at 1000 x g for one hour. The CF method was essentially the same as that of the laboratory branch complement fixation (LBCF) (3) except that C'titration was performed in the presence of test antigen. Five –50% hemolytic units of guinea pig complement were used.

Immunodiffusion (ID) Test

The antigens that were prepared for the CF test were also tested for precipitating antigen in agar gel double diffusion tests against rabbit RP antiserum (as described by White) (11) and PPR goat immune serum in addition to SPC convalescent sera to test the antigenic relationship between RP, PPR and SPC viruses. Known PPR, RP and normal control antigens were also included in series. These were similarly prepared from LN tissues of goats (PPR) or bovines (RP).

Cross Neutralization (CN) Test

The cross neutralization test was adopted to study the immunological relationship between the SPC, the PPR and the RP viruses. Normal control and convalescent or immunized goat sera against SPC, PPR and RP viruses were tested for neutralization against each of the three viruses. The SPC virus source was a cell culture harvest of fourth virus passage in Vero cells. Heat-inactivated sera diluted 1:5 in PBS were further diluted into twofold dilutions. The various serum dilutions were mixed with equal volumes of virus suspension containing approximately 100 ID_{50}, and the mixtures were allowed to stand at 37°C for 30 minutes. Each virus was mixed with equal volumes of PBS to serve as virus controls.

Two-tenths ml of each mixture was inoculated onto triplicate confluent Vero cell cultures grown on 50 ml Falcon plastic flasks. The initial serum dilution without virus was included as serum control. The cultures were incubated at 37°C and examined periodically under a light microscope for the development of CPE. The endpoint neutralization was considered as the highest serum dilution that neutralized the virus in more than one-half of the cultures tested. Virus neutralization was also conducted at a constant dilution of serum (1:5) against serial tenfold dilutions of each virus. Neutralization indices were expressed as logs of virus neutralized by each serum.

Challenge with RP Virus

Three goats which survived the infection with SPC virus were challenged intramuscularly with 1 ml of RP virus of the Kabete "O" virulent strain that contained 1 x $10^{4.5}$ $TCID_{50}$. Two normal control goats received the same amount of virus to test the infectivity of the challenge virus. The animals were kept under observation for three weeks.

Electron Microscopy (EM)

Vero cell cultures inoculated with BCC from goats that were experimentally infected with SPC specimens and that revealed CPE were repassed in Vero cell cultures and processed for EM. Control and infected Vero cell cultures were fixed *in situ* in 1% (wt/vol) glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.2 for 15 minutes at 4°C. After this initial fixation, the cells were gently scraped from the plastic surface with a rubber policeman and centrifuged at 500 x g for five minutes to form a soft pellet. This pellet was washed three times in cold 0.1M cacodylate for an hour at 4°C. The cells were then postfixed in 1% osmium tetroxide in 0.1M cacodylate buffer, then dehydrated in a graded ethyl alcohol-propylene oxide series, embedded in Epon 812[4] and polymerized at 60°C for 24 hours. For immunoelectron microscopy (IEM), the cells were gently scraped in the media and lightly centrifuged into a soft pellet. The pellet was resuspended in 0.2 ml of homologous goat serum, PPR or RP sera that were previously heat inactivated (56°C for 30 minutes) and absorbed by normal

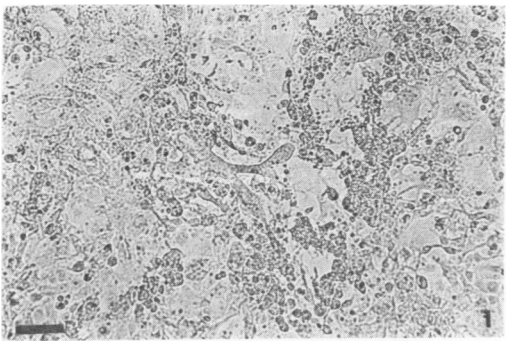

Fig. 1. Photomicrograph of Vero cell culture 8 days postinoculation with blood from goat inoculated with stomatitis pneumoenteritis complex goat tissue suspension. Cell culture shows cytopathic effect. Bar = 25 μm.

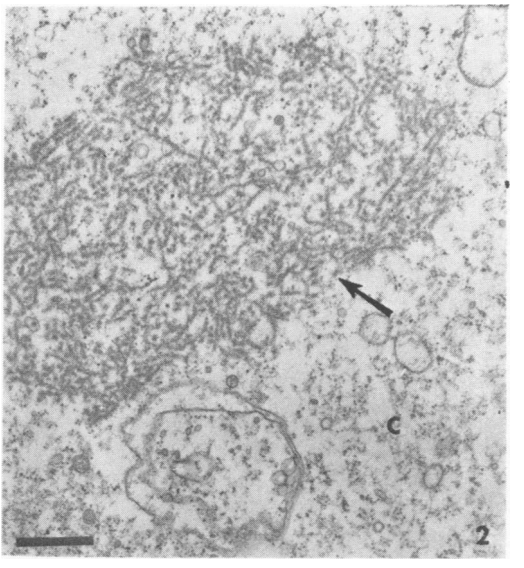

Fig. 2. Cytoplasmic inclusion made up of random array of fibrillar strands (arrow) presumably viral ribonucleoprotein. Bar = 1 μm.*

Vero cell culture pellet. The cell suspensions were incubated for 30 minutes, washed three times in phosphate buffer then allowed to incubate with ferritin conjugated rabbit antigoat immunoglobulin (1). After the cells were washed three times in phosphate buffer, they were fixed in 1% glutaraldehyde, postfixed in 1% osmium in phosphate buffer dehydrated and embedded as described above. Thin sections were cut on a Porter Blum MT-2 Ultramicrotome (Sorvall, Inc., Norwalk, Conn.) and stained

*Figures 2 to 7 are electron micrographs of thin sections of Vero cell infected with stomatitis pneumoenteritis virus, isolated from goat blood, fixed with glutaraldehyde-osmium and stained with uranyl acetate. N = nucleus, NM = nuclear membrane, PM = plasma membrane and C = cytoplasm.

TABLE I. Response of Goats to Exposure with Stomatitis Pneumoenteritis Complex Virus

No. of Animal	Age[a]	Treatment[b]	Duration of fever	Mouth Lesions	Diarrhea	Respiratory signs	Virus recovery	CF antigens in LN[c]	Antibody detection	Termination	Response to RP Virus challenge 6 weeks after Exposure
			(days)								
3968.....	B	Inoc. IM	6	+	+	+	+	+	−	Death 11 DPI	NA
3964.....	A	Contact	6	−	+	+	+	NT	+	Recovery	Survival
3965.....	B	Inoc. IM	4	++	+	+	++	+	−	Death 11 DPI	NA
3345.....	A	Contact	5	++	+	+	++	NT	+	Recovery	Survival
3967.....	B	Inoc. IM	0	−	−	−	−	NT	+	Recovery	Survival
3341.....	A	Contact	6	+	+	+	+	+	−	Death 20 DPE	NA
3996.....	Y	Inoc. IM with 1st gt. passage	4	+	+	+	+	+	−	Death 12 DPI	NA
3997.....	Y	"	7	+	+	+	+	+	−	Death 20 DPI	NA
3998.....	Y	"	7	+	+	+	+	+	−	Sacrificed at moribund	NA
C#1.....	Y	−	−	−	−	−	−	−	−	−	Death 9 DPI
C#2.....	Y	−	−	−	−	−	−	−	−	−	Death 9 DPI

[a]B: baby, A: adult, Y: yearling
[b]Inoc. IM: inoculation intramuscular
[c]CF: complement fixation, LN : lymph node, NT: not tested
DPI: days postinoculation, DPE: days postexposure, Rp: rinderpest, NA: not applied

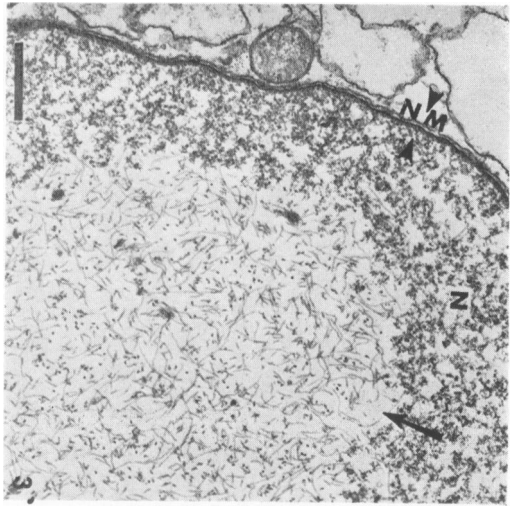

Fig. 3. Intranuclear inclusions (arrow) made up of random array of fine fibrillar strands. Both longitudinal and transverse sections are observed. Bar = 1 μm.

with saturated aqueous solution of uranyl acetate and lead citrate (8). Electron micrographs were taken with an RCA EMU-3G electron microscope.

RESULTS

Clinical Picture

Results in Table I show the response of goats to inoculation with tissue suspensions from Nigerian diseased goats and also of goats exposed by close contact to the inoculated kids. All of these goats developed similar signs and lesions.

The incubation period in inoculated goats lasted four to six days. The onset of illness was manifested by the initial appearance of watery ocular and nasal discharge, followed by mucopurulent discharge as the disease signs progressed. Fever of more than 40°C persisted for five to seven days. Nasal discharge was pronounced after the onset of fever and persisted for two to seven days. Mucosal erosions three to four days after the onset of fever were seen on the lips, gums, cheeks and corners of the mouth. The tongue had thick, white necrotic areas. Erosions were seen when the necrotic membranes were removed. At the end of fever, the lips were matted and the eyelids stuck together. The nasal opening was covered with mucopurulent discharge

that flowed over the muzzle. The muzzle was covered with dried, crusty discharge. Affected animals had diarrhea after the temperature decline and became weak; and death followed prostration. The incubation period in the contact goats was two to three days longer than in the inoculated goats. However, they demonstrated the same disease signs and one of them died (Table I). All affected goats coughed; coughing began with the appearance of nasal discharge.

Necropsy revealed erosions and ulcerations of the mucosa of the digestive tract especially of the gums, inner lips, cheeks and hard palate and severe hemorrhagic inflammation of the abomasum. Linear hemorrhages were seen in the cecum and colon and along the folds of the rectum (zebra stripes). Erosions of the turbinates, tracheitis and pneumonia were seen in the respiratory tract.

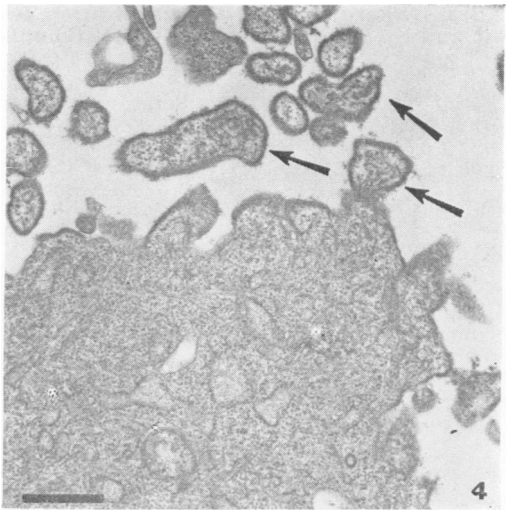

Fig. 4. Extracellular pleomorphic virions (arrows) **assuming different sizes and shapes. Bar = 1 μm.**

Virus Isolation

A cytopathic virus was isolated in Vero cell cultures from the BCC preparation of goats inoculated with SPC virus infected tissue suspensions (Fig. 1). The CPE was characterized by cell rounding, clumping into grape-like clusters, syncytia formation and the appearance of stellate or spindle cells with long, fine, often anastomosing processes. The CPE started as confined foci in some areas of the cell sheet then generalized and spread outwards from the initial foci. The CPE was similar to that developed in PPR virus infection. (F. M. Hamdy, A. H. Dardiri and S. S. Breese, Jr. 1974, unpublished data.)

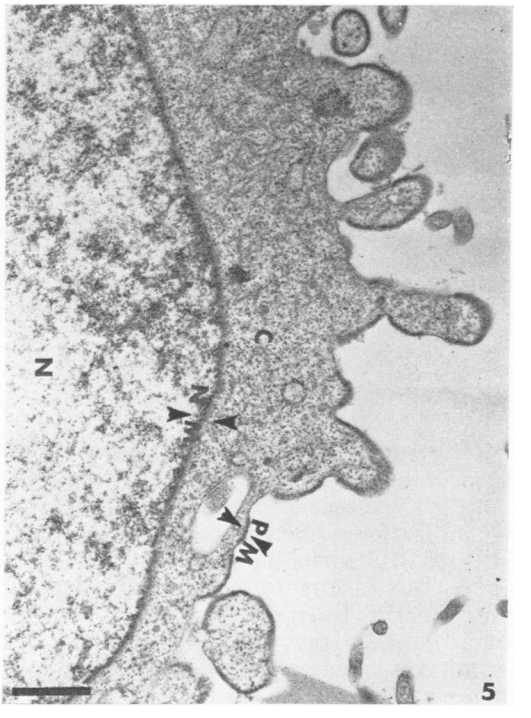

Fig. 5. Virus particles are seen budding from the plasma membranes of infected cell. Modified plasma membrane at areas containing viral envelope is more thickened than that in the other areas of the plasma membrane of the cell. Bar = 1 μm.

CF Test

Lymph nodes from dead goats that had been inoculated with SPC specimen revealed CF activity with RP and PPR antiserum and against the homologous system. The CF titers were 20, 80 and 80 respectively as calculated by net weight of original lymph node tissue. Normal antigen and serum controls were negative.

Immunodiffusion

Two precipitate lines were observed between the central well that received lapinized RP serum and each of the wells that received RP, PPR and SPC virus antigens. Precipitate lines were also noted between these three antigens and the central well receiving goat PPR immune serum or SPC convalescent goat serum. Normal serum and antigen controls did not show precipitate lines.

TABLE II. Results of Cross Neutralization Tests Between Peste des Petits Ruminants, Rinderpest and Stomatitis Pneumoenteritis Complex Viruses[a]

Virus	Sera[b] PPR Virus	RP Virus	SPC Virus	Control	Virus	Sera[c] PPR Virus	RP Virus	SPC Virus	Control
PPR	5.0	0.5	5.0	0.5	PPR	640	<10	640	<10
RP	0.5	5.0	0.5	0.3	RP	<10	640	<10	<10
SPC	5.0	0.5	5.0	0.5	SPC	640	<10	640	<10

[a]All numbers represent the mean of at least six serum titers
[b]Numbers represent neutralization indices expressed as number of $\log_{10}$ of virus completely neutralized by serum
[c]Numbers represent the dilutions of sera that neutralized 100 $TCID_{50}$ of virus in more than half of the cultures tested

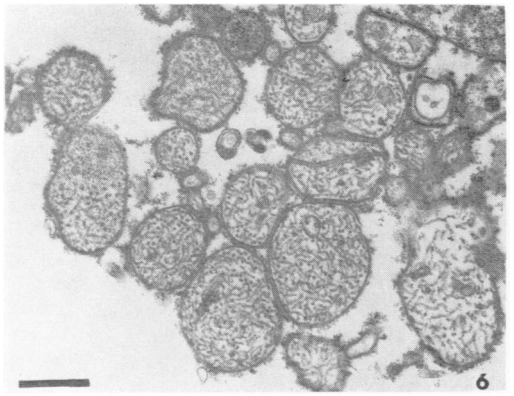

Fig. 6. Extracellular virions from specimen treated with homologous goat serum and ferritin-conjugated rabbit antigoat immunoglobulin. Note the tagging of ferritin particles around the viral envelopes. Bar = 0.5 μm.

Cross Neutralization

Results of neutralization tests revealed reciprocal cross neutralization between SPC and PPR viruses but revealed no significant neutralization occurring between either the SPC or PPR virus and RP virus. Table II shows the results of cross neutralization between SPC isolate, PPR and RP viruses. Data indicate that PPR virus and SPC isolate are identical to each other but are different from RP virus.

Infectivity of the Cell Culture Propagated Viral Isolate (3968 Vero 4) to Susceptible Goats

Inoculated goats as well as goats exposed by close contact developed clinical signs and lesions as summarized in Table III. The signs and lesions were similar to those produced by field materials (Table I) and field observations.

Ultrastructural Morphology

Electron microscopic examinations of thin sections of Vero cells infected with the second passage of SPC isolate revealed virus and virus-related morphological structures identical to those of PPR virus. (F. M. Hamdy, A. H. Dardiri and S. S. Breese, Jr., 1974 unpublished data.) Figures 2 and 3 are two electron micrographs showing intracytoplasmic and intranuclear inclusions respectively. Virus budding was observed at the plasma membrane of the infected cells. Mature virions were seen extracellularly (Fig. 4).

Immuno-electron Microscopy

Thin sections of Vero cells infected with SPC viral isolate treated with homologous, PPR, or RP goat antisera and ferritin conjugated rabbit antigoat immunoglobulins were examined by electron microscopy. Ex-

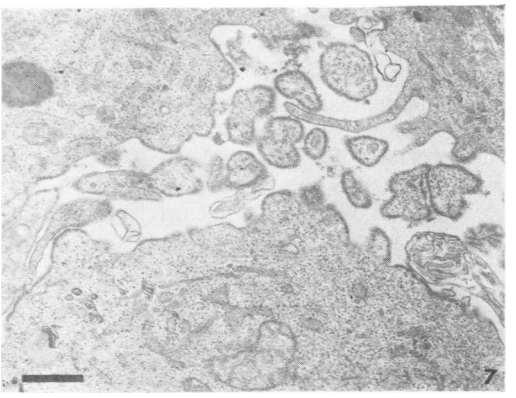

Fig. 7. Extracellular virions from specimen treated with rinderpest immune goat serum and ferritin-conjugated rabbit antigoat immunoglobulins. Note that tagging is much less than that in the homologous system. Bar = 1 μm.

TABLE III. Response of Goats to Cell Culture Propagated Stomatitis Pneumoenteritis Complex Viral Isolate (3968 Vero 4)

Goat No.	Treatment	Clinical Signs	Virus Recovery	Antibody Development	Termination	Response to Subsequent RP Virus Challenge
4364	Inoculated IV	+	+	+	Recovery	Resist
4365	"	+	+	−	Death 9DPI	NA
4366	"	+	+	+	Recovery	Resist
4367	"	+	+	+	Recovery	Resist
4368	Close Contact	+	+	+	Recovery	Resist
4369	" "	+	+		Recovery	Resist

IV: intravenously. DPI: days postinoculation, NA: not applied

amination revealed equal tagging in specimens treated with the homologous serum and PPR antiserum and significantly less tagging in RP antiserum treated samples. Figures 5 to 7 represent some of the observations of this experiment. Tagging was observed to occur at the surfaces of the enveloped virions as well as in some antigenic sites of the modified plasma membrane of the infected cells and in the immature virus structures at the budding stages.

Resistance of Surviving Goats to RP Virus Challenge

The three goats that survived the infection, either through inoculation or close contact, resisted challenge with 1 x $10^{4.5}$ $TCID_{50}$ of virulent RP virus whereas the two control goats inoculated with the same dose of RP virus died nine DPI with classical signs and lesions of RP.

Examination for other Microorganisms

The results of examination of the SPC virus infected cell culture harvest were negative for mycoplasma, bacteria and fungi. The attempts for virus isolation on nine day old chicken embryos and other cell cultures were negative.

DISCUSSION

The similarity of clinical signs and macroscopic lesions caused in American Goats by PPR virus and the SPC virus isolate of Nigeria suggests that the two syndromes represent the same disease and are caused by the same virus. The inability to isolate organisms other than PPR virus such as mycoplasma from the cell culture harvest that reproduced the disease indicates that PPR virus is the sole cause of this disease.

The spread of the disease from baby goats to adult goats indicates that the SPC is contagious. This finding is in agreement with that of Nduaka and Ihemelandu (6) who reported that this condition was contagious in the field.

In this study, the similarity of clinical pictures, postmortem lesions, cytopathology produced by SPC and PPR viruses in Vero cells, the ultrastructural morphology and the serological identity in CF, ID, CN and IEM and the resistance to RP virus challenge of survived goats gave evidence of identity. The induction of the disease by virus isolate propagated in cell culture and the recovery of a virus with PPR virus characteristic from blood of the sick goats demonstrated PPR virus etiology of this syndrome.

With regard to the relationship with RP virus, PPR shows some antigenic determinants in common causing cross reactions in the ID and CF tests. These common antigens could possibly be of ribonucleoprotein origin and not of the structural proteins of the viral envelope. This hypothesis would explain the dissimilarity in virus neutralization because neutralization occurred only in the homologous system and not in the heterologous system.

With regard to control of the disease, investigations in this laboratory (2) revealed that tissue culture propagated attenuated RP virus protected goats against virulent PPR as evidenced by resistance to challenge with virulent virus in spite of lack of specific PPR virus neutralizing antibody in their sera. The duration of this immunity is yet to be determined.

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