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Proteins Specified by African Swine Fever Virus

II. Analysis of Proteins in Infected Cells and Antigenic Properties

By

E. Tabarés, J. Martinez, F. Ruiz Gonzalvo, and C. Sánchez-Botija

Departamento de Virología Animal, Instituto Nacional de Investigaciones Agrarias, Madrid, Spain

With 8 Figures

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Summary

Infection of MS cells with African swine fever virus (ASFV) produces inhibition of protein synthesis which is detectable from 4.5 hours after infection. At least 34 viral polypeptides have been indentified with molecular weights ranging between 9500 and 243,000 daltons. Three of these proteins show affinity for the cell nucleus and nine are in both the nuclear and cytoplasmic fractions. Ten early proteins were found, and most of the structural proteins were late proteins. Most of the proteins are synthesized within the first 8 hours after infection.

At least nine proteins induced antibodies in the natural infection. Six of these proteins are structural proteins. The antigenic determinants of VP172, VP162, VP146, VP73, VP34, and IP23.5 are in the primary structure of the proteins.

Introduction

African swine fever virus (ASFV) is an icosahedral cytoplasmic deoxyvirus, tentatively classified as an iridovirus (1). Information on the replication of ASFV has been obtained mainly from electron microscopic studies of virus infected cells (4, 13, 18). Biochemical studies of the infection have been concerned primarily with DNA synthesis (12, 15, 20). The proteins which induce antibodies in the natural infection have been partially characterized (6).

The present work deals with the kinetics of protein synthesis in ASFV infected cells, localization of proteins within the cell, and classification of early and late proteins as well as their antigenic properties. Furthermore, we studied the effect of sodium dodecyl sulfate (SDS) on the antigen-antibody reaction and characterized the antigenic determinant structure.

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Materials and Methods

Cells and Virus

Spain-70 (E-70) strain of ASFV was used after 40 passages in the MS monkey kidney cell line. The virus was titrated in pig leukocyte cultures by the immunofluorescent technique (20).

Labelling of Infected Cells by Isotopes

The kinetics of virus protein synthesis were determined in the presence of 2 μg/ml of mitomycin C added to the cells 12 hours before infection and in presence or absence of 200 μg/ml sodium phosphonoacetate (PAA) added at the time of infection. Infected cells grown in Dulbecco's modified Eagle medium (DEM) and supplemented with 1 per cent calf serum were pulse-labeled for 2 hours with 5 μCi/ml of ^{35}S-methionine (358 mC/mmol). PAA was a gift from Prof. Y. Becker, the Hebrew University, Jerusalem.

Preparation of Whole Cells and Nuclear and Cytoplasmic Fractions

After periods indicated in the text 1 mg of methionine was added, the radioactive medium was removed, and the cells were washed with cold 0.34 M sucrose, 0.005 M Tris-HCl pH 7. The cells were collected in 0.067 M sucrose, 0.005 M Tris-HCl pH 7 using a rubber policemen (total fraction). The cells were allowed to swell for 10 minutes at 0° C and then lysed by the addition of Nonidet P-40 (NP-40) to a final concentration of 1 per cent (w/v) for 10 minutes at 0° C. Nuclei were sedimented at $1000 \times g$ for 6 minutes at 4° C onto a 0.34 M sucrose, 0.005 M Tris-HCl pH 7 cushion. The total, nuclear, and cytoplasmic fractions were then solubilized for electrophoresis.

Preparation of Cytoplasmic Fraction

Infected cells were labeled with 2 μCi/ml of ^{35}S-methionine for 24 hours after infection in DEM without methionine supplemented with 2 per cent calf serum. Cells were washed with PBS and harvested at 48 hours p.i. and then sedimented at $650 \times g$ for 5 minutes. The pellet was washed with 0.34 M sucrose in 0.005 M Tris-HCl pH 8, allowed to swell in 0.067 M sucrose in 0.005 M Tris-HCl pH 8 for 8 minutes at 0° C, and was then lysed by addition of NP-40 to a final concentration of 1 per cent (w/v) for 10 minutes at 0° C with occasional agitation. Sucrose was added to obtain a final concentration of 8 per cent (w/v), and the nuclei were pelleted at $1000 \times g$ for 10 minutes at 0° C. The cytoplasmic fraction was made in 0.002 M EDTA, 0.05 M β-mercaptoethanol in the presence or absence of 0.5 NaCl. After 15 minutes at 25° C, the mixture was centrifuged at $100{,}000 \times g$ for 1 hour at 4° C and the clear layer between an upper lipid layer and bottom precipitate served as the cytoplasmic fraction for the immunoprecipitation reaction. Uninfected cells were treated in the same way and served as control antigen.

Preparation of Staphylococcus aureus

A heat-killed suspension of protein A-bearing *Staphylococcus aureus* (strain Cowan I gift of Dr. N. Rubio, C.S.I.C. Madrid) was prepared (9). Prior to use the bacteria were washed in 0.1 M NaCl, 0.5 per cent (w/v) NP40, 2 mM EDTA, 5 mM β-mercaptoethanol and 0.05 M Tris pH 8 (CNTE buffer) and resuspended in the same buffer to a final concentration about 20 per cent (v/v) adjusted with the aid of a hematocrit centrifuge.

Condition for Immunoprecipitation

The cytoplasmic fraction (50 μl) was incubated for 1 hour at 25° C in CNTE buffer (500 μl) with or without SDS and either normal or infected pig serum (10 μl). Washed protein A-bearing *S. aureus* (50 μl) was then added and the material was incubated for an additional period of 15 minutes at 25° C. Bacteria were centrifuged at $2000 \times g$ for 10 minutes at 4° C and washed in CNTE buffer and phosphate buffered saline. The final bacterial pellets were resuspended in 200 μl of electrophoresis sample buffer

[0.05 M Tris-HCl pH 7, 2 per cent sodium dodecylsulphate (SDS), 5 per cent β-mercaptoethanol, 0.01 per cent bromophenol blue and 20 per cent (v/v) glycerol] and incubated at 60° C for 5 minutes. After pelleting the bacteria the supernatant fluids were heated to 100° C for 2 minutes and subjected to electrophoresis through polyacrylamide slab gels.

Acrylamide Gel Electrophoresis

All procedures and fluorography were previously described (19).

Results

Analysis of Proteins Synthesized in Infected Cells at Different Times Post-Infection

We have measured the synthesis of proteins after virus infection by incorporation of ^{3}H-methionine at different times post-infection. Fig. 1 indicates the incorporation of ^{3}H-methionine. Inhibition of the synthesis of proteins in the infected cells was observed from 4.5 hours post-infection.

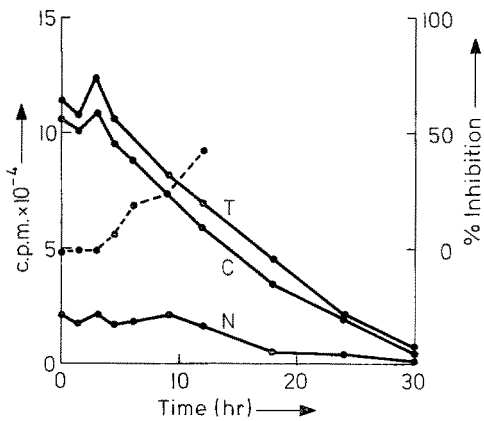

Fig. 1. Incorporation of ^{3}H-methionine in ASFV infected cells. At various intervals after infection, cells were incubated with 9 μCi/ml of ^{3}H-methionine (specific activity 250 mCi/mmol) for 30 minutes. Cells were disrupted in hypotonic buffer and the analysis was done as described previously (20). The radioactivity incorporated into the total fraction (T), nuclear fraction (N) and cytoplasmic fraction (C) was determined (—) and the percentage inhibition (- - -) of cellular protein synthesis. Percentage inhibition was calculated as: $\left(1 - \frac{ANI}{AI}\right) \times 100$. Where ANI (activity of non-infected cells) and AI (activity of infected cells) respectively are given as cpm ^{3}H-methionine/μg protein

Analysis of polypeptides in MS cells infected with ASFV by high-resolution polyacrylamide gel electrophoresis revealed the synthesis of at least 34 polypeptides (IP) ranging in molecular weight from 9500 to 243,000 daltons (Fig. 2).

To show categorically that a protein is virus specified, it must be synthesized in a cell system incorporating free amino acids under the control of virus specific

mRNA. Since this is not feasible at present, we defined an infected cell polypeptide (IP) if it met one or more of the following criteria:

i) Stimulation in the rate of synthesis after infection;
ii) immuno precipitation of IP by antisera against viral antigens;
iii) differences in electrophoretic mobility.

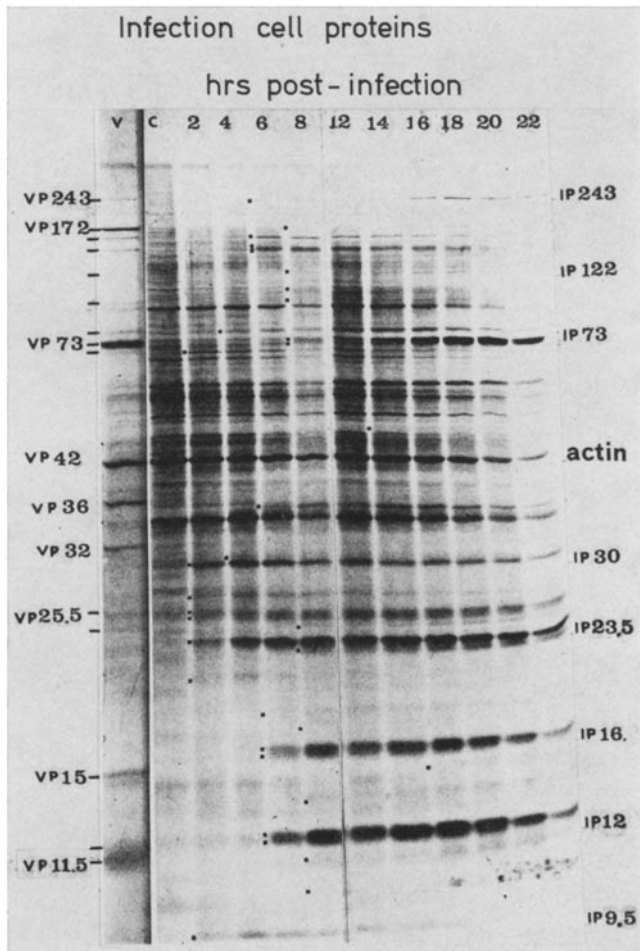

Fig. 2. Sequential synthesis of ASFV induced proteins in MS cells. The cells were infected with about 100 FC D 50/cell. At the times indicated (hours p.i.) the cells were labeled for 2 hours with ^{35}S-methionine. Samples were electrophoresed in 15 per cent acrylamide gels. *V* viral structural proteins and *C* uninfected cell control. Appearance of the protein (·) Viral structural protein identified (—)

We have defined the IP as structural (S) or non structural (NS) polypeptides on the basis of their electrophoretic mobility when compared with polypeptides from purified virus pending a more complete characterization of these proteins.

The polypeptides that are not well defined were designated unassigned (U) polypeptides.

Based on these criteria it will be shown that there are a minimum of 14 structural, 18 non-structural, and 2 unassigned polypeptides made during the productive infection of MS-cells with ASFV (Table 1). The rest of the structural polypeptides are not well defined since their electrophoretic mobilities may differ from that of their precursors (VP36 is probably the IP35). They represent cell components which become part of the structure of the virion (for example actin), or they are masked by cell polypeptides with the same electrophoretic mobility.

Table 1. *Infected cell polypeptides synthesized at various times after infection*

Band No.	IP mol. wt. $\times 10^{-3}$	Relationsh. to viral prot.	Early proteins	Distribution in cell	Cytoplasmic antigens	Kinetics of appearance
1	243	S[1]		N/C[4]		4—6
2	172	S		N/C	+	6—8
3	162	S		N/C	+	4—6
4	146	S		N/C	+	4—6
5	144	NS[2]	+ (U)[3]	U		4—6
6	122	S		C[5]		6—8
7	104	NS		C		6—8
8	97	S		C		6—8
9	80	S		C		2—4
10	73	S		N (N/C)	+	6—8
11	71	NS		N (N/C)		6—8
12	63	S		C		0—2
13	48	U (S)		U		12—14
14	35	U (S)		N/C		4—6
15	31	NS	+	C	+	2—4
16	30	NS	+	C	+	0—2
17	27	NS	+	C		0—2
18	25.5	S	+	C		0—2
19	25	NS	+	C		0—2
20	24.5	S		C		6—8
21	23.5	NS	+	N/C	+	0—2
22	23	NS		N/C		6—8
23	20.5	NS		U		0—2
24	19	NS		C		4—6
25	18	NS		U		6—8
26	16.5	NS		N[6]		4—6
27	16	NS		N		4—6
28	15	S		U		14—16
29	14.5	NS		C		6—8
30	13	NS	+	C		4—6
31	12	S	+	C	+	4—6
32	11.5	S		C		6—8
33	10	NS		N		6—8
34	9.5	NS	+	C		0—2

[1] structural, [2] nonstructural, [3] unassigned, [4] nuclear/cytoplasmic, [5] cytoplasmic, [6] nuclear.

Molecular weights of IP obtained by interpolation of previously determined values for structural viral polypeptides (19). See text for criteria applied in the classification

The appearance of the IP components is sequential (Table 1) and most of them are synthesized within the first 8 hours after infection. The polypeptides may be divided into the following categories on the basis of their distribution in the cell after 2 hour pulses (Fig. 3): i) Predominantly nuclear IP (73-71, in the first hours; 16.5, 16, and 10.0); ii) Nuclear/cytoplasmic IP (243, 172, 162, 146, 73—71, 35, 23.5, and 23 in the last hours); iii) predominantly cytoplasmic IP (122, 104, 93, 80, 63, 31, 30, 26.9, 25.5, 25.0, 24.5, 19, 14.0, 13, 12, 11.5, and 9.5). IP 243 may exist in the nuclear fraction at 20 hours *p.i.* due to the breakdown of the nuclear membrane during the course of the infection.

The nuclear fraction was isolated in the presence of 1 per cent (w/v) NP-40. The absence of actin was a criterion for the absence of cytoplasm in such frac-

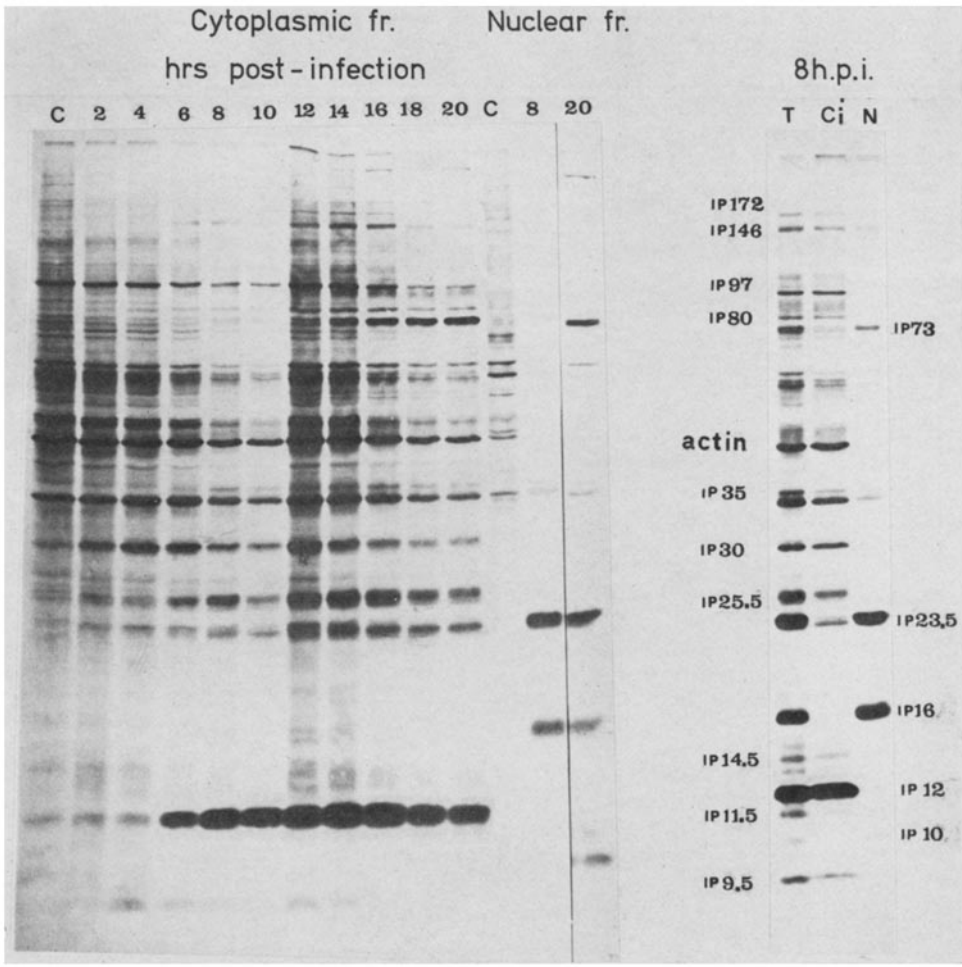

Fig. 3. The distribution between nucleus and cytoplasm of labeled infected cell polypeptides. Uninfected and infected MS cells were labeled with ^{35}S-methionine for 2 hours at the times indicated (hours p.i.) and processed for electrophoresis and fluorography. Numbers indicate hours after the pulse. *C* control uninfected cells; *T* total fraction; *Ci* cytoplasmic fraction; *N* nuclear fraction

tions. Furthermore some proteins were found only in the cytoplasmic fraction which further indicates the degree of purity of the nuclear fraction.

Some nuclear proteins may be involved in viral DNA synthesis (20).

Identification of Early Proteins

Phosphonoacetic acid (PAA) was used as a selective inhibitor of viral DNA synthesis (12). Confluent monolayers of MS cells were exposed to 200 μg/ml of

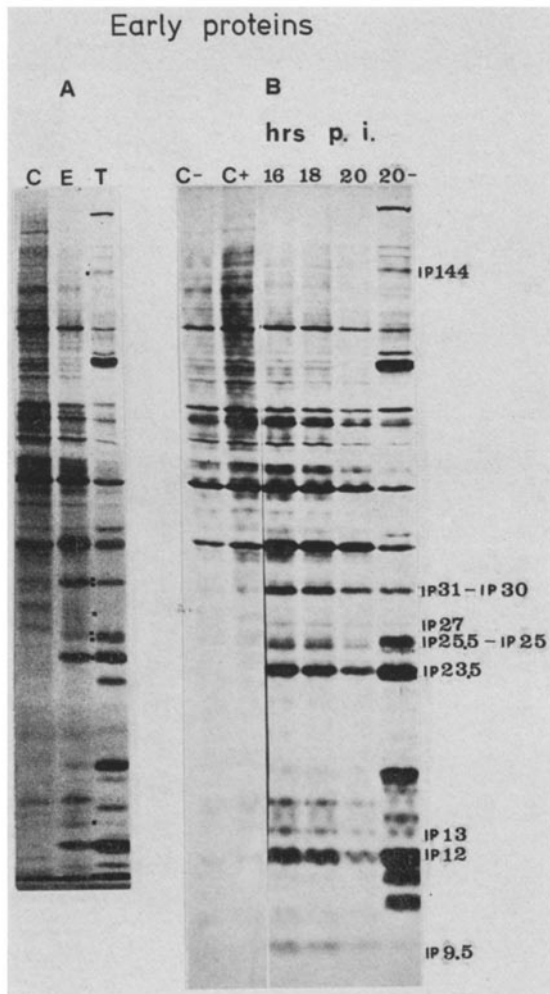

Fig 4. Fluorography showing early proteins in MS cells infected with ASFV. *A* Distribution of proteins at 20 hours p.i.; *C* control normal uninfected cells; *E* infected cells with PAA; *T* infected cells without PAA. *B* At times indicated (hours p.i.) the cells infected in the presence of 200 μg/ml of PAA were labeled for 2 hours with ^{35}S-methionine. *C*— control normal uninfected cells without PAA; *C*+ control normal uninfected cells with PAA. The concentration of acrylamide in the gel A was slightly less than in gel B and the proteins with small molecular weights moved further, allowing the separation between IP 23.5 and IP 23.0

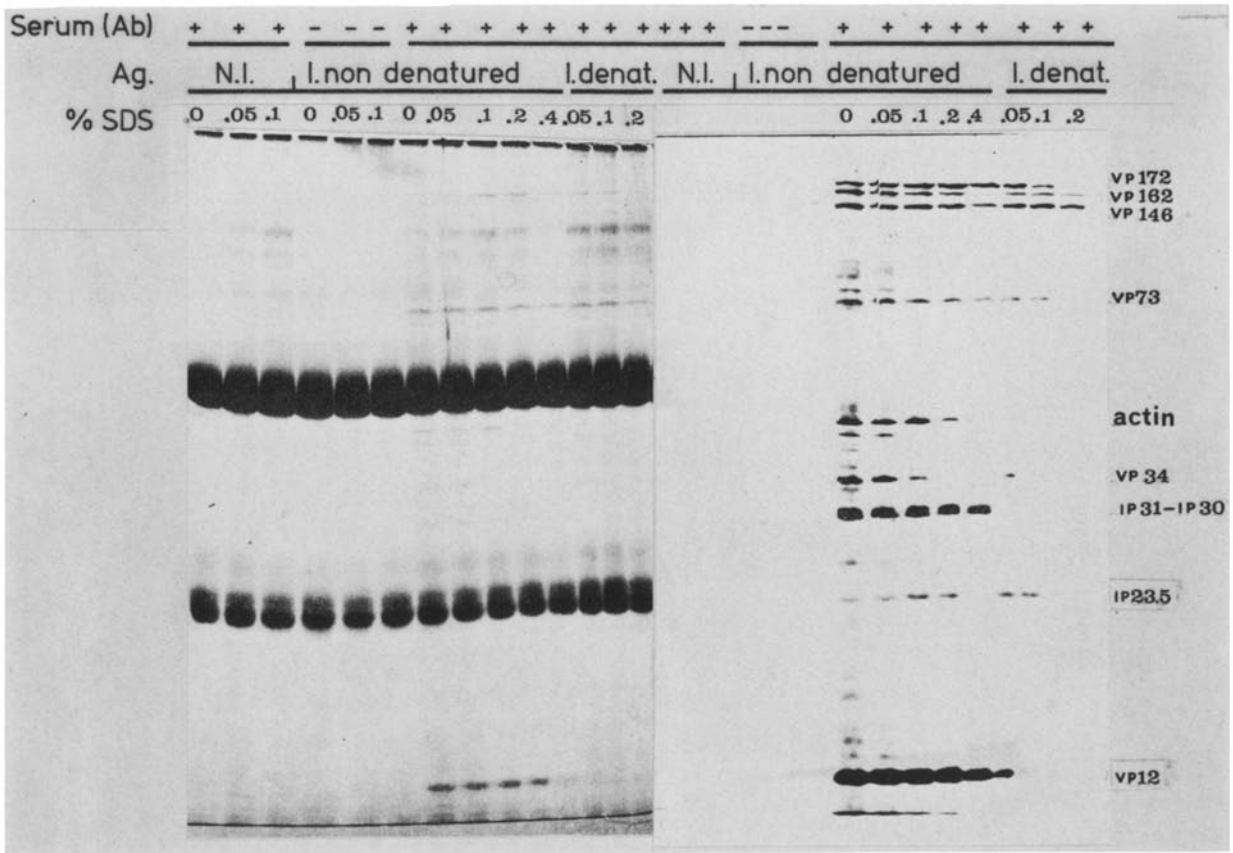

Fig. 5. Antigens of African swine fever virus in natural infections. Coomassie brilliant blue staining (left) and fluorography (right) of immunocomplex polypeptides. These were obtained by reaction between cytoplasmic antigens (isolated without NaCl) and hyperimmune serum. *I* cytoplasmic antigen from infected cells; *NI* from non-infected cells; *serum* + or − with or without antibodies against ASFV; %*SDS* concentration of SDS present in the antigen-antibody reaction

PAA from the time of infection. Under these conditions, ten polypeptides, IP144, IP31, IP30, IP27, IP25.5, IP25, IP23.5, IP13, IP12, IP9.5, were synthesized in the absence of viral DNA synthesis and were defined as early proteins (Fig. 4). Most of the structural polypeptides are synthesized as late proteins (IP243, IP172, IP162, IP146, IP122, IP97, IP80, IP73, IP63, IP24.5, IP15, IP11.5).

Identification and Characterization of Viral Antigens

Up to now 5 specific antigens have been identified by cross electrophoresis in infected cells (6), although they may not have been sufficiently characterized. In cross electrophoresis, each antigen may consist of several polypeptides (14) which makes identification of the true antigenic polypeptide difficult.

The Cowan I strain of *Staphylococcus aureus* was used as an adsorbent for antibodies complexed with radio-labeled antigens from cytoplasmic cell fractions. Although the cytoplasmic fractions after treatment with detergent were centrifuged at 100,000 $\times g$ (see Material and Methods), some nonantigenic proteins may

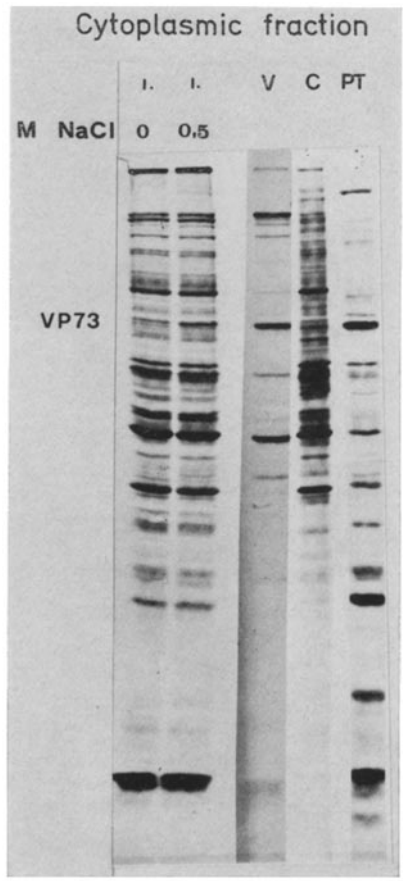

Fig. 6. Fluorography of polypeptides of the cytoplasmic fraction. Cytoplasmic fractions used in immunoprecipitation: *I* antigen isolated from infected cells with 0.5 M NaCl (0.5) and without NaCl (0); *V* viral structural polypeptides; *C* control normal uninfected cells and *PT* total proteins in infected cells

have sedimented with the antigenic proteins, thus complicating the identification of the antigens.

To increase the solubilization rate, the cytoplasmic material was treated with SDS. Several treatments were done with different concentrations of SDS with and without thermic denaturation. The antigen-antibody reaction was carried out at different SDS concentrations to eliminate nonespecific reactions. The final products of the reaction between cytoplasmic antigens (extracted without NaCl) and hyperimmune pig serum were analysed by electrophoresis with the following results (Fig. 5): There was no reaction between the hyperimmune serum and cytoplasmic antigens from non-infected cells and between non-infected normal pig serum and the cytoplasmic antigens from infected cells. The nonspecific background proteins decreased with the increase in SDS concentration in the reaction. We have estimated the optimal concentration of SDS as being between 0.1 and 0.2 per cent.

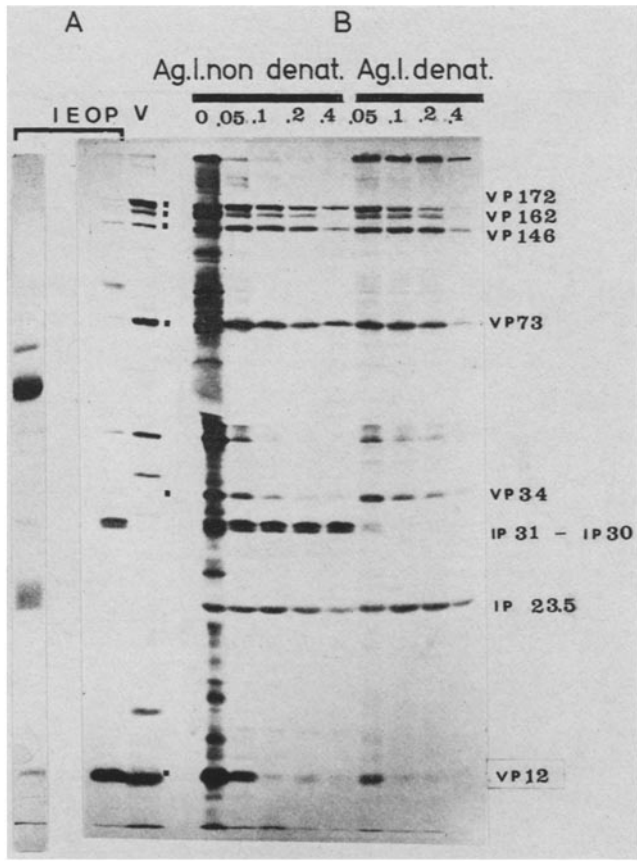

Fig. 7. Antigens of African swine fever virus in natural infection. *A* Coomassie brilliant blue staining and fluorography of polypeptides of immunoprecipitate after immunoelectroosmophoresis (IEOP), *V* Fluorography of viral structural polypeptides. *B* Fluorography of immune complexes, obtained by reaction between cytoplasmic infected fraction (obtained in 0.5 M NaCl) and hyperimmune serum. The numbers indicate the per cent SDS present in the reaction

Under these conditions ten polypeptides react, one of them having the same electrophoretic mobility as actin. This protein may be the viral protein with the same electrophoretic mobility as actin since the cellular actin from non-infected cells does not react. Also the actin may be associated with antigen IP 12.0 since it seems that they always react together. This association could not be destroyed by the action of SDS without destroying the antigenic determinant. Quantitatively protein IP12.0 occurs in the largest amount which seems to indicate that it is the strongest inducer for antibodies in the natural infection.

Antigenic determinants of IP31, IP30, and VP12 are affected by thermic denaturation with SDS and β-mercaptoethanol suggesting that the antigenic determinants are not located in the primary structure.

The same reaction was performed with cytoplasmic antigens extracted in the presence of 0.5 M NaCl yielding higher amounts of VP73 (Fig. 6). In this experiment the antigen was treated with 2 per cent (w/v) SDS and than diluted to the reaction concentrations (Fig. 7). The antigenic determinant of the protein IP12.0 was affected indicating its high sensitivity to the detergent.

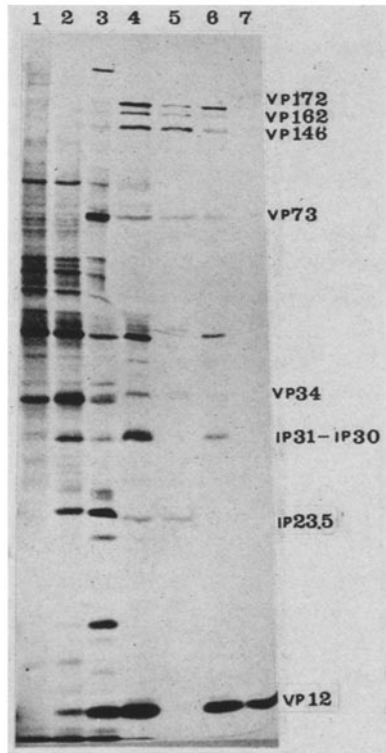

Fig. 8. Antigens of African swine fever virus in natural infection. Fluorography between cytoplasmic infected fraction (isolated without NaCl) and several pig sera with specific antibodies against ASFV (4, 5, 6, 7). In 5 the cytoplasmic infected fraction was denatured thermally with SDS. Uninfected cell protein *(1)*, uninfected cell proteins grown in presence of PAA *(2)* and infected cell proteins *(3)* are also shown. The fluorography of immune complexes was obtained by reaction between cytoplasmic infected fraction (obtained without NaCl) and the experimental sera

Of these nine proteins (not including VP42) at least five of them are clearly viral structural proteins: VP172, VP162, VP146, VP73, and VP34. The proteins IP31, IP30, and IP23.5 are nonstructural proteins. The IP12.0 may be a minor component of the intracellular virus with the same electrophoretic mobility as VP12. This protein is the major antigen in the immunoelectroosmophoresis (IEOP) (Fig. 7), a technique widely used in diagnosis (16), which indicates that it is the strongest inducer for precipitating antibodies in the natural infection. The antigen with the electrophoretic mobility corresponding to the VP34 may be specifically viral, although its synthesis may be masked by a cell protein.

The specificity of the cytoplasmic antigens was also verified by reaction with specific ASF antibodies in different experimental sera (Fig. 8). The level of reaction of each antigen depends on the serum type.

Discussion

At least 34 polypeptides induced in ASFV infected MS cells have been detected according to their electrophoretic mobility. Of these 14 have been identified as structural proteins and two were not allocated since they may not be structural proteins. There are minor structural components which may be cellular or may be masked by the synthesis of cell proteins with the same electrophoretic mobility.

Synthesis of the different polypeptides is initiated sequentially and then are synthesized within the first 8 hours after infection. This agrees with the observation by electron microscopy of viral structures in infected cells between 6 and 10 hours postinfection (13, 18). Synthesis of most of the viral proteins at 8 hours p.i. coincides with the increase in titre of intracellular virus (5, 20). Some specific viral proteins migrate to the infected cell nucleus and seem to be involved in viral DNA replication (20). These results agree with the detection by immunofluorescence of viral antigens inside the infected cell nucleus (IC Plan, personal communication). These proteins may also be involved in some changes detected in the chromatin structure (13).

It is possible to differentiate between early and late proteins in the infected cells which are not found in frog virus 3 (FV_3) infected cells (8) although FV_3 is structurally analogous to ASFV and is classified in the same family. Most of the structural proteins are late proteins as found also with the vaccinia virus (17), which assembles in the cytoplasm too. The formation of viral structures in infected cells treated with hydroxyurea agrees with the previous finding that at high concentrations of hydroxyurea some infectious virus is produced indicating that viral DNA synthesis is not completely inhibited (4). The appearance of viral late proteins between 4 and 6 hours p.i. coincides with the start of viral DNA synthesis (20, 21).

Characterization of the antigenic proteins is of great importance from the point of view of improving the specificity of existing diagnostic techniques and clarifying the immunological role they play in immunity. This is of great importance especially since neutralizing antibodies have not yet been detected in pigs although resistance against homologous virulent virus does exist (7).

Up to now at least 5 antigens were known to produce antibodies in the natural infection (6). Nevertheless the structural polypeptides of these antigens were not characterized. In this study at least 10 antigenic proteins have been demonstrated, 6 of them being characterized as structural proteins and 3 as non-structural. The last one is not clearly defined.

The antigenic protein with a molecular weight of 12,000 induces the greatest amount of antibodies and it may be of great importance for the detection of antibodies in pigs infected with ASFV, since the IP12.0 is an early protein produced without requiring viral DNA synthesis (i.e. without virus production).

Although the influence of SDS in the antigen-antibody reaction has already been studied (2, 10, 11), we have assayed new concentrations in order to increase the specificity of the reaction. Greater specificity was found with concentrations of SDS below 0.2 per cent (w/v), and adsorption of the antigen-antibody complex to protein A of *Staphylococcus aureus* was not affected in the presence of 0.4 per cent (w/v) SDS. Denaturation of the polypeptides with SDS, β-mercaptoethanol and temperature enabled us to establish that the antigenic determinants of the polypeptides VP172, VP146, VP73, VP34, and IP23.5 are in the primary structure. Knowledge of the structure of the antigenic determinants is essential. for the isolation and purification of antigens.

Acknowledgments

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Authors' address: Dr. E. TABARÉS, Departamento de Virologia Animal, Instituto Nacional de Investigaciones Agrarias, c/Embajadores, 68, Madrid-12, Spain.

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