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Monoclonal Antibodies of African Swine Fever Virus: Antigenic Differences among Field Virus Isolates and Viruses Passaged in Cell Culture

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An analysis of the binding properties of a collection of monoclonal antibodies to African swine fever virus particles showed that virus field isolates passaged in porcine macrophages changed antigenically more than a strain of a cell-adapted virus passaged in Vero cells. From seven clones isolated from the spleen of a field-infected pig, we found four clones that had the same antigenic properties, one clone that had large changes in proteins p150 and p27 and small changes in proteins p37 and p14, and two clones that had minor changes in proteins p150 and p27, respectively. An analysis of the binding properties of the monoclonal antibodies to 23 field isolates from Africa, Europe, and America showed that the African isolates differed among themselves more than the European and the American isolates; in this study we found changes in 8 of the 10 virus proteins tested. The most variable proteins in the African isolates were p150, p 27, p14, and p12. In contrast to the African isolates, protein p12 from the non-African viruses did not change. The clustering of the field virus isolates in six antigenic homology groups indicated the existence of a complex variety of African swine fever virus serotypes.

African swine fever (ASF) infects in vivo all the species of the *Suidae* (Artyodactyla: Suiformes) family that have been tested and in vitro porcine macrophages. After adaptation, several established cell lines can propagate the virus (47, 48).

The sera from pigs and other virus-resistant animal species inoculated with ASF virus do not neutralize the virus, although they contain virus-specific antibodies (11). A possibility to account for the lack of detection of neutralizing sera is antigenic variation of critical antigens. Indications that ASF virus undergoes antigenic alterations are the following: (i) pigs infected with attenuated viruses, derived from virulent ones by passage in porcine leukocytes in vitro, are partially resistant to the original but not to other virulent isolates (7, 13, 14, 22, 24, 32, 37, 41); (ii) a soluble antigen, isolated from the spleen of infected pigs, seems to be isolate specific (40); (iii) the hemadsorption-inhibition reaction (8, 45, 46), complement-mediated cytolysis (31), and a comparison of the DNA restriction patterns (44, 50, 51; R. Blasco, unpublished data) have made possible the distinction of several virus isolates.

Monoclonal antibodies (MAbs) are useful reagents to show antigenic variation of viruses (2, 16, 49, 52). The availability of a collection of MAbs which recognize 10 ASF virus proteins (39) has allowed us to (i) determine the antigenic changes of ASF virus passaged in vitro in either porcine leukocytes or monkey kidney (Vero) cells; (ii) show the existence in a single infected pig of viruses with different antigenic properties; and (iii) classify 23 field virus isolates into six antigenic homology groups.

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MATERIALS AND METHODS

Cells and viruses. Porcine blood monocytes and alveolar macrophages were obtained as described elsewhere (5, 15). Vero cells (CCL81) were obtained from the American Type Culture Collection (Rockville, Md.).

Table 1 indicates the nomenclature, the origin, and some properties of the ASF virus isolates used in this study. ASF virus adapted to grow in Vero cells and the conditions for virus titration and cloning have been described previously (15).

The cloning of each field virus isolate was carried out by endpoint dilution in porcine monocytes cultured in Microtest II plates (Becton Dickinson Labware, Oxnard, Calif.). About 2×10^4 cells attached to the bottom of each well were infected with serial virus dilutions. The presence of hemadsorbing virus was determined by rosette formation 3 to 5 days after the addition of porcine erythrocytes (25). Cytopathic, nonhemadsorbing virus was detected by cell detachment and lysis 5 to 6 days after infection. The virus from the cultures inoculated with the largest dilution that showed a positive hemadsorption or cytopathic effect was selected for the next cloning step. Each virus isolate was cloned at least three times. The hemadsorbing virus clone HIN54-HAD$^+$ and the nonhemadsorbing one HIN54-HAD$^-$ were isolated from the same virus stock (Table 1), and both kept their properties for at least three additional cloning steps.

Virus passages were done weekly by infection of either swine macrophages or Vero cells at a multiplicity of infection of about 10^{-3} hemadsorption units or PFU per cell, respectively.

Virus production and purification. Cloned field isolates of ASF virus were propagated in porcine alveolar macrophages cultured in spinner flasks as described by Carrascosa et al.

TABLE 1. Field isolates of ASF virus

Isolate[a]	Origin	Received from:	Material	Previous history	Reference
African					
SPE51	Rhodesia (Spencer)	R. Wesley	Spleen	Unknown	18
HIN54	Kenya (Hinde)	R. Wesley	Spleen	Pig passage no. uncertain	21
WH55	Uganda (wart hog)	R. Wesley	Spleen	Unknown	12
UGA59	Uganda	P. Wilkinson	Spleen	Pig passage no. uncertain	13
TEN61	Malawi (Tengani)	R. Wesley	Plasma	Pig passage no. uncertain	10
KIR69	Tanzania (Kirawira)	P. Wilkinson	Spleen	3 pig passages	19
European					
LIS57	Portugal (Lisbon)	J. Vigario	Spleen	2 pig passages	27
LIS60	Portugal (Lisbon)	J. Vigario	Spleen	2 pig passages	26
BA60	Spain (Badajoz)	A. Ordás	Buffy coat	3 leukocyte passages	36
BA71	Spain (Badajoz)		Spleen	See text	15
PO70	Spain (Pontevedra)	C. S. Botija	Buffy coat	Unknown	C. S. Botija, personal communication
B78	Spain (Barcelona)	A. Ordás	Serum	Unknown	A. Ordás, personal communication
CC83	Spain	A. Ordás	Spleen	Unknown	A. Ordás, personal communication
FRA64	France (Perpignan)	R. Wesley	Spleen	2 pig passages	20
ITA67	Italy	R. Wesley	Spleen	5 leukocyte passages; 2 pig passages	28
SAR78	Sardinia	P. Wilkinson	Spleen	Unknown	9
SAR82	Sardinia	P. Wilkinson	Spleen	Unknown	P. Wilkinson, personal communication
American					
BRA78	Brazil	R. Wesley	Spleen	1 pig passage	30
BRA79	Brazil	R. Wesley	Buffy coat	2 buffy coat passages; 2 pig passages	29
DR79	Dominican Republic	R. Wesley	Spleen	2 pig passages	29
HAI81	Haiti	R. Wesley	Spleen	1 buffy coat passage	C. Mebus, personal communication

[a] Each virus isolate is named by a code that indicates the origin and the isolation year.

(5). Vero cell-adapted virus was produced in Vero cell monolayers infected at a multiplicity of infection of about 0.1 PFU per cell. ASF virus was partially purified from the culture medium as described by Tabarés et al. (43). Sucrose gradient-purified Moloney leukemia virus was provided by J. H. Ihle (Frederick Cancer Research Center, Frederick, Md.).

MAbs. The MAbs used in this work recognized 11 ASF virus proteins and at least 20 different epitopes (39; A. Sanz, B. García Barreno, L. Enjuanes, and E. Viñuela, unpublished data). The protein A-binding MAbs were purified by protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) affinity chromatography (23). MAbs of the immunoglobulin G1 subclass were purified by ion-exchange chromatography in DEAE-Sephacel (Pharmacia) by the method of Parham et al. (34). The purified MAbs were labeled with ^{125}I as described by Greenwood et al. (17).

Binding assays. Partially purified ASF virus isolates were dissociated by treatment with 0.5% Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo.) and 0.5 M NaCl in phosphate-buffered saline (pH 7.2) for 1 h at 37°C. This procedure solubilized more than 95% of the major capsid protein p72. The dissociated viruses were diluted 100-fold in phosphate-buffered saline and bound to flexible polyvinyl microplates (Dynatech Laboratories, Inc., Alexandria, Va.) by overnight incubation at 4°C. Unspecific binding sites were blocked by overnight incubation with 5% bovine serum albumin (fraction V; Sigma) in phosphate-buffered saline for 2 h at 37°C.

The amount of each virus isolate was determined by a competitive radioimmunoassay for the ASF virus major protein p72, which remained essentially unchanged from one isolate to another (see Results). Twofold dilutions of detergent-disrupted virus isolate or homogeneous protein p72 (obtained by immunochromatography; C. Vela, L. Enjuanes, A. L. Carrascosa, and E. Viñuela, unpublished data), were used to block the binding of a limiting amount of ^{125}I-labeled, protein p72-specific MAb 1B.C11 to partially purified ASF virus BA71 bound to plastic plates. The results were normalized to 100% binding in the absence of competing antigens. All the binding assays were carried out with an amount of virus equivalent to 0.15 μg of protein p72 (Fig. 1), which gave a maximum binding of antibodies in the binding assays (data not shown).

The same kind of competitive radioimmunoassay was used to determine the antigenic variation of individual epitopes after a different number of virus passages in vitro. In this case the reference virus corresponded to passage 1 and the competition viruses to those obtained after different passages (see Fig. 3).

The binding of MAbs to the different virus isolates bound to polyvinyl plates was carried out by incubation with 50 μl of hybridoma culture supernatant for 1 h at 37°C. Unbound immunoglobulin was removed by washing with 0.1% bovine serum albumin–0.1% Tween 80 in phosphate-buffered saline (washing buffer). In the case of nonadapted virus produced in macrophages, ^{125}I-labeled sheep anti-mouse $F(ab')_2$ immunoglobulin (specific activity, 6 μCi/μg; 4×10^5 cpm per well; New England Nuclear Corp., Dreieich, Federal Republic of Germany) in 50 μl of washing buffer containing 0.1% nonimmune serum was added, and the incubation was continued overnight. Unbound radioactive material was eliminated with washing buffer, and the radioactive material in each

well was determined with a gamma spectrometer (Wallace, model 80000; LKB, Bromma, Sweden). In the case of ASF virus adapted to grow in Vero cells, the binding assay described by Sanz et al. (39) was used.

The binding percentage value of each MAb to each virus isolate relative to the binding to a reference virus was calculated by using the formula: $[(C_{ij} - C_{lj})/(C_{il} - C_{ll})] \times 100$, where C_{ij} = counts per minute of MAb i bound to virus isolate j; C_{lj} = counts per minute of a blank MAb l (an MAb specific for protein p30 from Moloney leukemia virus) bound to virus isolate j; C_{il} = counts per minute of control MAb bound to the reference virus isolate; and C_{ll} = counts per minute of control MAb bound to the reference virus isolate; values were determined in triplicate samples.

Antigenic homology. The percentage of antigenic homology of a particular virus isolate relative to the reference virus BA71 was estimated by the formula: $[(0 \times a + 1 \times b + 2 \times c)/(2 \times 42)] \times 100$ where $(a + b + c = 42)$, where a, b, and c are the number of MAbs with binding percentage values equal to 0 to 20, 21 to 40, and 41 to 100, respectively, for the considered virus isolate, and 42 is the total number of MAbs. The MAbs with a binding percentage lower than 20% (type *a*) do not contribute to the overall homology percentage, whereas MAbs with binding values of 21 to 40 and 41 to 100 were arbitrarily weighted to values of 1 and 2 to obtain the final homology percentage. The estimate of the percent homology between the virus isolate being tested and the standard, reference virus was determined by comparing the binding of 42 MAbs. For example, if 1 MAb (*a*) had 16% binding, a second (*b*) had 28% binding, and the remaining 40 (*c*) had over 80% binding, according to the above formula the unknown isolate would have $[(0 \times 1 + 1 \times 1 + 2 \times 40)/(2 \times 42)] \times 100 = 96.4\%$ antigenic relatedness. To determine the ASF virus isolates included in a group with a predetermined percent homology, each virus isolate was compared with all other isolates without establishing a priori a reference virus. All the viruses showing a percent homology equal to or larger than 90% were included in the same group.

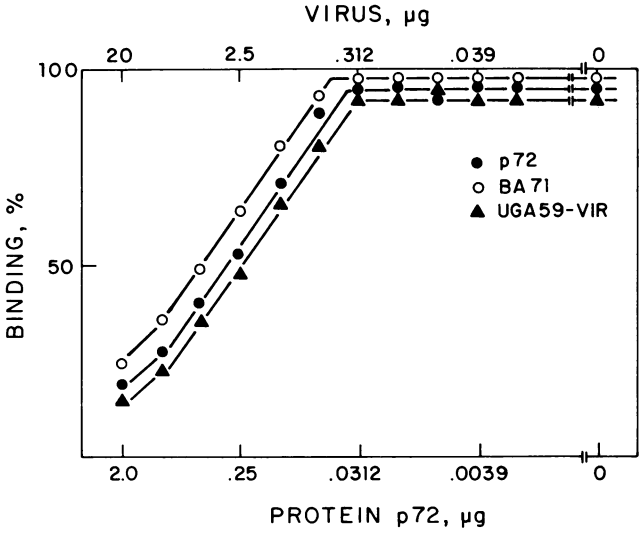

FIG. 1. Determination of protein p72 in partially purified preparations by a competitive radioimmunoassay. Twofold dilutions of ASF viruses UGA59 and BA71 or homogeneous major capsid protein p72 in 0.5 M NaCl–0.1% Nonidet P-40 were used to block the binding of ^{125}I-labeled, protein p72-specific MAb 1B.C11 to partially purified ASF virus BA71-V bound to polyvinyl chloride plates. After the incubation the wells were washed with washing buffer, and the radioactive material in each well was determined with a gamma spectrometer.

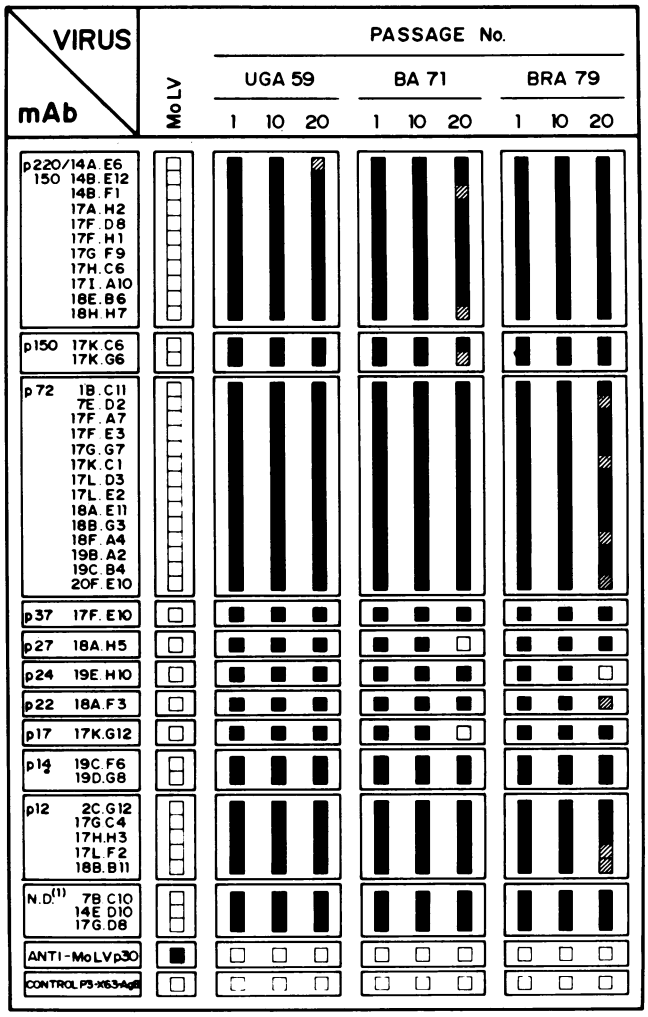

FIG. 2. Binding of MAbs to ASF virus isolates passaged in porcine macrophages. The value of the MAb binding to the viruses after passage 1 was taken as the reference value (100). Symbols: □, 0 to 19; ▨, 20 to 40; ■, 41 to 100. MoLV, Moloney leukemia virus. N.D., Not determined.

RESULTS

Antigenic variation of ASF virus passaged in cell cultures. (i) Field isolates passaged in porcine macrophages. Figure 2 shows the changes in the binding of 42 ASF virus-specific MAbs to three virus isolates (UGA59, BA71, and BRA78; Table 1) after 10 and 20 passages in porcine macrophages derived from blood monocytes. The three viruses did not change between passages 1 and 10, but between passages 11 and 20, they showed differences from the original viruses. Of the 10 proteins recognized by the MAbs, 8 had changed, and the percentage of antigenic homology of each virus with respect to the original virus was 99, 92, and 89% for UGA59, BA71, and BRA78, respectively. The major antigenic changes took place in proteins p27, p24, and p17.

When the binding of a MAb to a particular virus passage decreased, the change of the epitopes in different virus proteins was evaluated by competing the binding to the

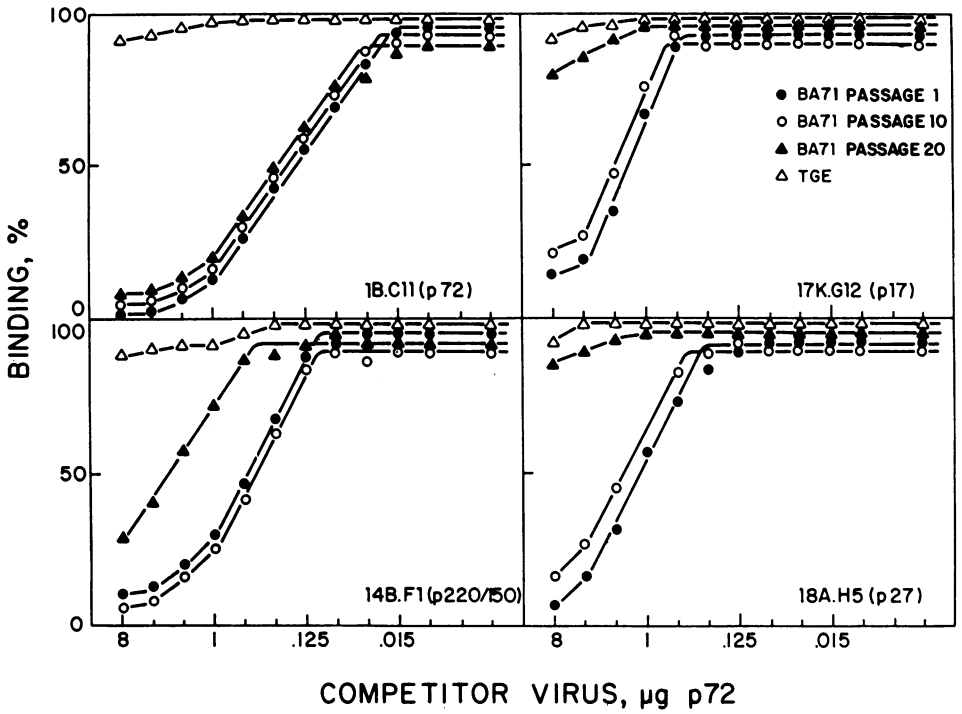

FIG. 3. Epitope variation of ASF virus passaged in porcine macrophages tested by a competitive radioimmunoassay. TGE, Transmissible gastroenteritis virus.

original virus clone with the viruses obtained after different numbers of passages in porcine macrophages (Fig. 3).

(ii) Adapted virus passages in Vero cells. Figure 4 indicates the passage history of ASF virus adapted to grow in Vero cells (BA71-V). After 16 passages in Vero cells, an analysis of nine clones did not show any change, but after passage 140, analysis of 13 clones showed that one of them had changed epitopes in proteins p150, p27, and p17 and another had changes in protein p27.

Antigenic differences among ASF virus clones isolated from a single pig. Figure 5 shows the binding properties of seven clones isolated from the spleen of an infected pig. Clone 4 had, in proteins p220/p150, p37, and p14 and in an unknown virus component, epitopes which were different from those present in the other clones. Clones 6 and 8 showed minor differences in proteins p220 and p27, respectively. The percentage of antigenic homology of clone 3 with the reference virus was 63%.

Antigenic differences among ASF virus field isolates. (i) African isolates. Figure 6 shows the binding properties of a panel of MAbs to eight African isolates relative to the binding to ASF virus BA71. The eight isolates were classified in four groups: I (SPE51, WH55), II (HIN54-HAD$^+$, UGA59-VIR), III (HIN54-HAD$^-$, UGA59-ATT, KIR69), and IV (TEN61), with the isolates within the same group showing a percentage of homology equal to or larger than 90%. The largest variability was shown by proteins p220, p150, p27, p14, and p12 and by some unknown virus com-

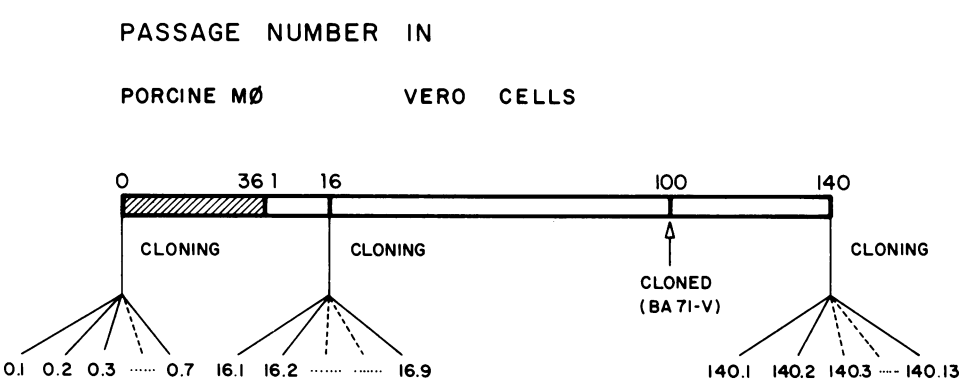

FIG. 4. Passage history of ASF virus adapted to grow in Vero cells. An ASF virus isolate obtained from the spleen of an infected pig in Badajoz (Spain) in 1971 was passaged in porcine macrophages 36 times. Vero cells were infected with virus from passage 36, and virus was propagated 100 times more in those cells. At passage 100 the virus was cloned to obtain BA71-V, the prototype virus, which was passaged 40 times more. Clones from passages 16 and 140 were obtained by plaque isolation in Vero cells.

ponent(s) recognized by MAbs 7B.C10, 14E.D10, and 17G.D8. Protein p72, the major capsid protein, did not change.

(ii) European and American isolates. Figure 7 shows the binding properties of the panel of MAbs to 10 European and 4 American virus isolates relative to the binding to ASF virus BA71. The European isolates were classified in four groups: I (BA71, CC83, SAR78, LIS60), II (FRA64, ITA67, PO70, B78, SAR82), III (LIS57), and IV (BA60). The proteins showing the largest antigenic differences were p220 and p150, but they were not, in quantitative terms, as large as those shown by the African isolates. The European virus isolates, in contrast with the African isolates, did not show any change in protein p12.

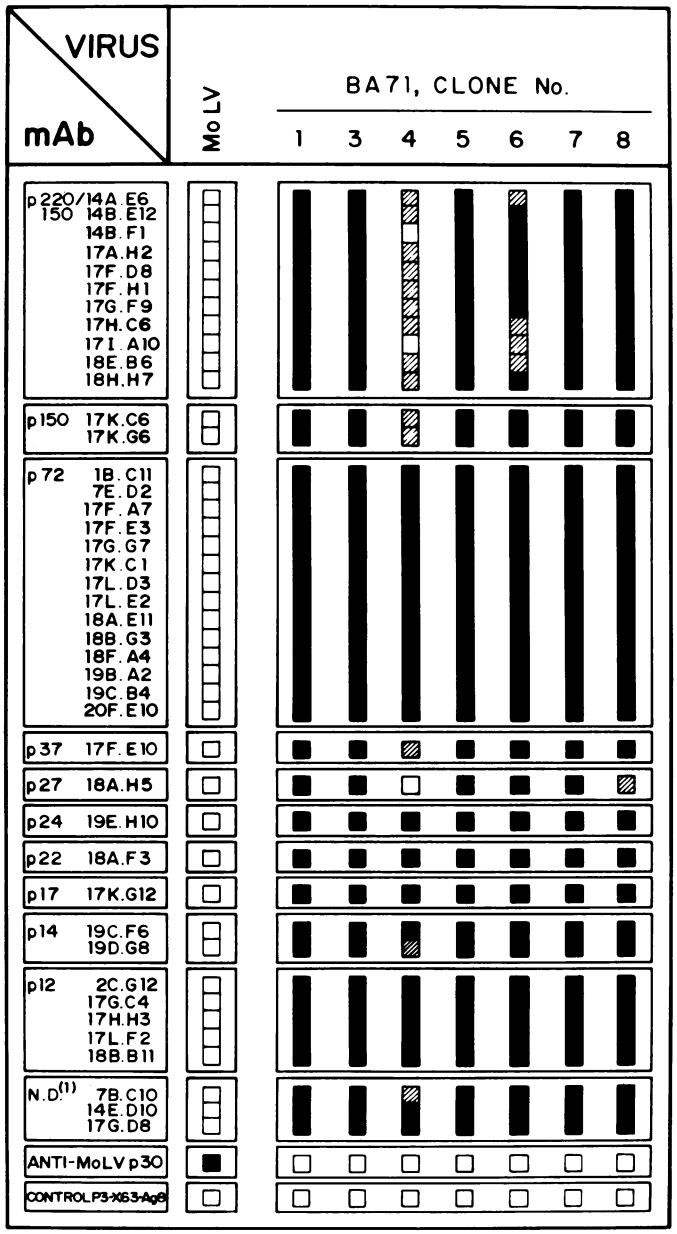

FIG. 5. Binding of MAbs to ASF virus clones isolated from the spleen of a pig infected in the field with BA71. Symbols are as described in the legend to Fig. 2. MoLV, Moloney leukemia virus. N.D., Not determined.

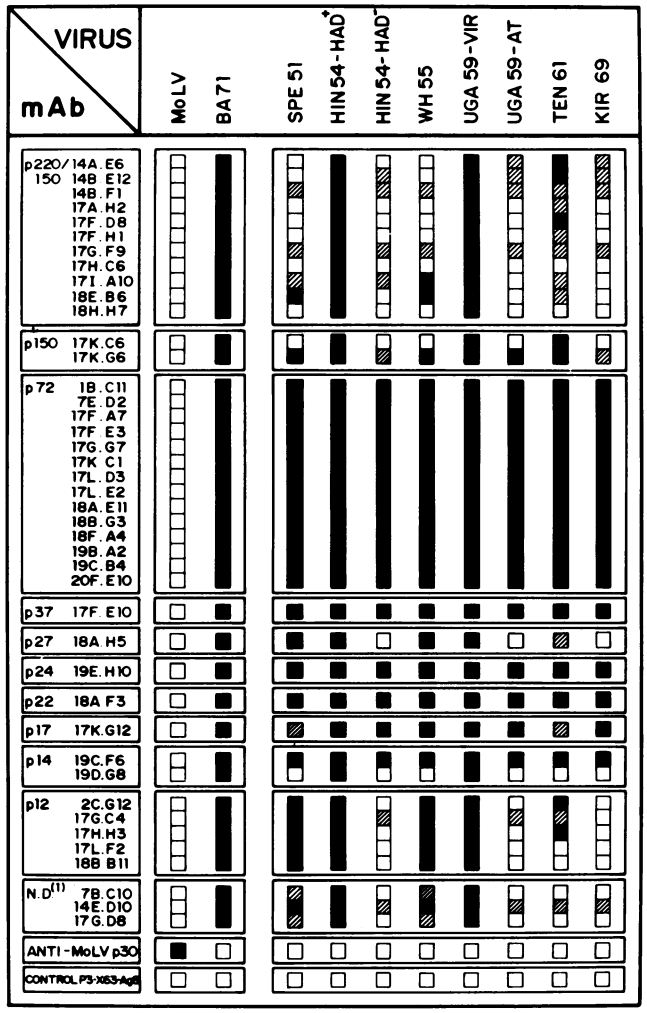

FIG. 6. Binding of MAbs to African ASF virus isolates. Symbols are as described in the legend to Fig. 2. MoLV, Moloney leukemia virus. N.D., Not determined.

The European isolates were classified into two groups: I (BRA79) and II (BRA78, DR79, HAI81). The American groups I and II were similar to the European groups I and II, respectively. The pattern of protein variation of the American isolates was similar to that of the European isolates.

Antigenic homology groups. Table 2 shows the six distinct serological groups of antigenic homology formed by ASF virus isolates with a homology greater than 90% among the isolates of each group, based on the binding of the MAb collection.

DISCUSSION

ASF virus replicates in vitro in pig monocytes and macrophages (25) and, after adaptation, in established cell lines from swine or animal species resistant to the virus (15, 33, 35). We showed that ASF virus field isolates passaged in porcine macrophages 10 to 20 times changed antigenically more than a strain of cell-adapted virus passaged 40 times in Vero cells (Fig. 2 and 3). In the latter case, the changes were seen only after clone analysis. In contrast, in the case of the virus passaged in macrophages, the changes could be seen in

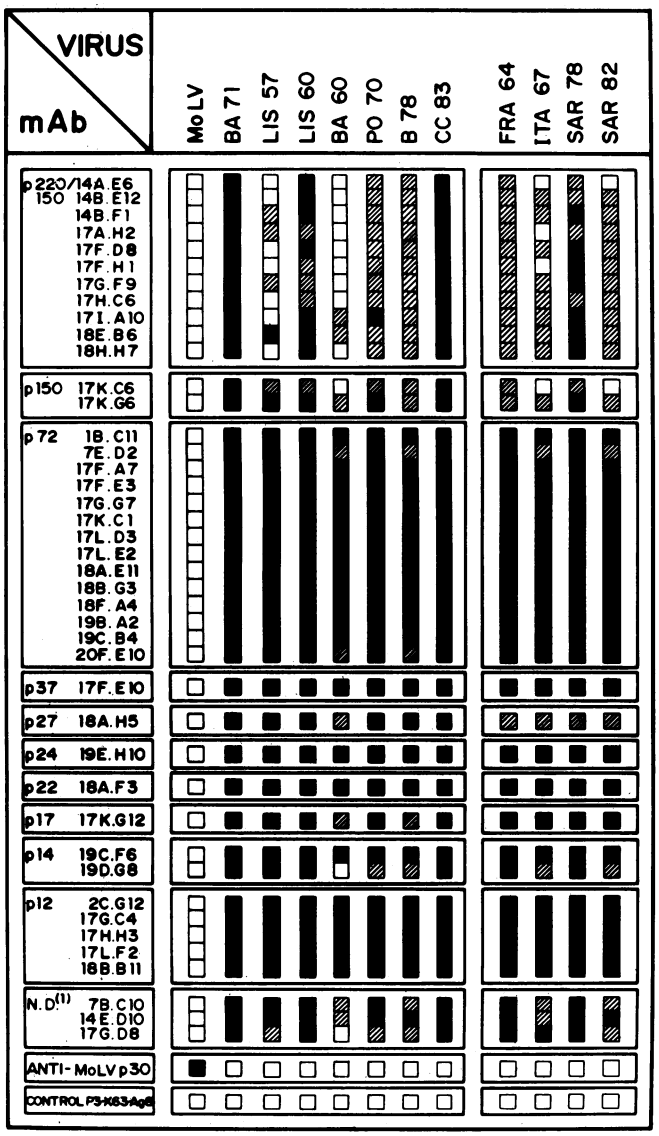

FIG. 7. Binding of MAbs to European and American ASF virus isolates. Symbols are as described in the legend to Fig. 2. MoLV, Moloney leukemia virus. N.D., Not determined.

uncloned samples. These differences could be due to the fewer number of passages of the field isolates (in one case, isolate BA71 was passaged in vitro only five times) or to the cell line used in the passages of the cell-adapted virus (1). The proteins that changed more frequently in both field isolates and cell-adapted virus passaged in vitro were p27, p17, and, to a lesser extent, p24.

An analysis of seven clones isolated from the spleen of a pig infected in the field with ASF virus BA71 showed four clones that were identical, one clone with large changes in protein p150 and p27 and small changes in proteins p37 and p14, and two clones that had minor changes in proteins p150 and p24, respectively (Fig. 5). This result indicated that a naturally infected pig contained a collection of antigenic variants. The significance of this observation is uncertain, because it is unknown whether that animal had been infected by one or more viruses. The infection of pigs with cloned virus should allow determination of the antigenic variability of ASF virus in vivo.

The possibility that the incomplete neutralization of ASF virus by polyclonal antibodies (11) and MAbs (L. Enjuanes, M. L. Nogal, and E. Viñuela, unpublished data) was due to antigenic variability is an open question.

An analysis of the binding properties of the MAbs to 23 cloned field isolates from Africa, Europe, and America showed that the African isolates differed among themselves more than the European and the American isolates (Fig. 6 and 7). This is probably due to the fact that in Africa the virus has been circulating for a long time and has diverged extensively. The uniformity of the non-African isolates relative to the African ones might be due to the possibility that only one or few viruses entered Europe during 1957 to 1960 (26, 27, 36).

From a stock of hemadsorbing ASF virus HIN54 (21), we isolated a nonhemadsorbing clone (HIN54-HAD$^-$) by limiting dilution. Clone HIN54-HAD$^-$ showed intermediate or no binding to most of the p150- and p12-specific MAbs and no binding to other MAbs specific for proteins p27 and p14. In contrast, the hemadsorbing virus stock bound all MAbs, as could be expected for a mixture of both hemadsorbing and nonhemadsorbing viruses. Hemadsorption of swine erythrocytes to ASF virus-infected cells is due to a late function (3, 25), but the virus component involved in the reaction is unknown, and there are some indications of genetic instability of the hemadsorbing character (7). The serological identity of clones HIN54-HAD$^-$ and UGA59-ATT and that shown by the corresponding isolates HIN54-HAD$^+$ and UGA59-VIR was unexpected.

In field virus isolates we detected antigenic changes in 6 of the 10 ASF virus proteins for which there are available MAbs, suggesting a broad distribution of the variability. The most variable proteins in the African isolates were p150 and p12. Protein p150 is localized in the nucleoid and in a corner of the virus particle (6) and is antigenically related to protein p220 (39), a virus-induced, nonstructural protein that seems to be inserted in the membrane of infected cells (J. F. Santarén, unpublished data). The cell surface location of protein p220 may be related to its large variability. In contrast with the African isolates, protein p12 from the non-African viruses did not change. Protein p12 is a major structural protein, localized near the virion periphery (4, 6).

The antigenic stability of protein p72 is important because it is the main antigen used in the detection of ASF virus-specific antisera by an enzyme immunoassay for the diagnosis of infected pigs (38, 42; C. Vela, L. Enjuanes, S. Sanz, and E. Viñuela, unpublished data).

The significance of the changes of proteins p37, p27, p24,

TABLE 2. Distribution of ASF virus field isolates in antigenic homology groups[a]

1	2	3	4	5	6
FRA64	HIN54-HAD$^+$	HIN54-HAD$^-$	SPE51	TEN61	BA60
ITA67	UGA59-VIR	UGA59-ATT$^+$	WH55		
PO70	LIS60	KIR69	LIS57		
B78	BA71				
BRA78	SAR78				
DR79	BRA79				
HAI81	CC83				
SAR82					

[a] The virus isolates in the same group had an antigenic homology greater than 90%.

p17, and p14 and the unknown components recognized by MAbs 7B.C10 and 17D.G8 is uncertain because of the small number of MAbs available. Changes in proteins p37 and p24 were found only in field isolates passaged in swine macrophages. The change in the protein p24 epitope recognized by MAb 19E.H10 which was observed after passaging ASF virus BRA79 in swine macrophages was an unexpected observation because it is likely that protein p24 is a cell protein (39). If protein p24 is indeed a host protein, a possible explanation to account for its variation is that the amount of p24 in the virion depends on its interaction with some other virus component which is really the one that changed. In this case, the low binding of the p24-specific MAb would be due to a limiting amount of protein p24.

With the data available no clear separation of virus isolates from different continents could be established, since virus isolates from Africa, Europe, and America were present in the same antigenic homology group.

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