Porcine Leukocyte Cellular Subsets Sensitive to African Swine Fever Virus In Vitro

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African swine fever virus infected most, if not all, of the macrophages (monocytes) and ca. 4% of the polymorphonuclear leukocytes from porcine peripheral blood. B and T lymphocytes, either resting or stimulated with phytohemagglutinin, lipopolysaccharide, or pokeweed mitogen, were not susceptible to the virus. All of the mitogens used inhibited African swine fever virus multiplication in susceptible cells. The number of virus passages in vitro and the virulence degree of the virus did not affect the susceptibility of porcine B or T lymphocytes to African swine fever virus.

African swine fever (ASF) is an important disease affecting domestic pigs and is produced by an icosahedral virus with a double-stranded DNA molecule of ca. 170 kilobase pairs and classified as a member of the family *Iridoviridae* (for a review, see reference 29). The virus induces antibodies that do not neutralize the infectious particle (8).

ASF virus is specific for pigs and related animal species and infects mainly, if not exclusively, peripheral monocytes and macrophages (7, 15, 19). In vitro, macrophages from several ASF virus-resistant animal species are also resistant to the virus (10).

Recently, from studies carried out on ASF virus-infected leukocytes, it was concluded that B lymphocytes are infected by the virus (30, 31). In contrast, other authors have suggested that only T lymphocytes are particularly at risk in infected animals (26). To clarify this discrepancy, we have analyzed ASF virus-infected porcine leukocytes for the presence of both virus antigens and lymphocyte markers on the same cell and for the presence of virus structures in various cell types observed by electron microscopy.

We show in this paper that most of the macrophages and ca. 4% of the polymorphonuclear leukocytes (PMNL) from porcine peripheral blood were susceptible in vitro to ASF virus, whereas B and T lymphocytes, either resting or stimulated with mitogens, were resistant to the virus.

MATERIALS AND METHODS

Animals and cells. Large White pigs weighing 25 to 40 kg were used.

Porcine leukocytes were isolated after sedimenting a mixture of 5 volumes of heparinized (40 IU/ml) blood and 1 volume of 6% (wt/vol) dextran (Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline (PBS) as described previously (5). Contaminating erythrocytes were lysed in 0.155 M NH_4Cl -0.1 mM EDTA-0.01 M KHCO_3 (pH 7.4) (25). PMNL were isolated from the leukocytes on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) as described previously (5) and were more than 96% pure. They were contaminated with less than 4% lymphocytes and 1% macrophages.

Pig kidney cells (PK-15) were provided by the American Type Culture Collection (CCL 33).

Viruses. Two ASF virus clones were used to infect porcine leukocytes. ASF virus BA71-5 was isolated from the spleen of an infected pig (9), passaged five times in leukocyte cultures to clone by limiting dilution, and then expanded. ASF virus BA71-100 was a clone selected after 100 passages in porcine leukocytes from a stock of virus obtained from the same spleen as BA71-5. ASF virus titrations were performed in macrophages as previously described (9). Titers were calculated by the method of Reed and Muench and expressed as hemadsorption units (HADU) per milliliter (23).

Porcine parvovirus, strain NADL-2, was obtained from The American Type Culture Collection (ATCC VR 742). The titer was determined by indirect immunofluorescence on infected PK-15 cells and expressed as 50% infective dose units per milliliter.

Mitogens. Phytohemagglutinin (PHA; Miles-Yeda, Rehovot, Israel) and pokeweed mitogen (PWM; GIBCO Laboratories, Grand Island, N.Y.) were used at a concentration of 1 and 10 $\mu\text{g}/\text{ml}$, respectively. Lipopolysaccharide (LPS) was purified from *Acinetobacter calcoaceticus* (strain sp. 199A; National Collection of Industrial and Marine Bacteria, Edinburgh, Scotland) by phenol-water extraction of bacterial membranes as previously described (28) and used at a concentration of 20 $\mu\text{g}/\text{ml}$. The mitogen concentrations used were those giving maximum stimulation in a blastogenic assay performed with porcine leukocytes as indicated elsewhere (11). The incorporation of [^3H]thymidine after stimulation of 5×10^5 cells per well with the optimal concentrations of PHA, PWM, and LPS was 60,000, 140,000, and 25,000 cpm/well, respectively, and the average radioactivity incorporated by the unstimulated cultures was ca. 3,000 cpm/well.

Cell culture and virus infection. Porcine leukocytes were cultured after two protocols, one for unstimulated and another for mitogen-stimulated leukocytes. In the first case, 20×10^6 leukocytes in a test tube with 5 ml of Dulbecco modified Eagle (DME) medium with 30% fetal swine serum were infected with ASF virus (multiplicity of infection [MOI], 5 HADU/cell). The virus was adsorbed during 2 h with shaking, the unadsorbed virus was removed by washing the cells three times with DME medium, and the cells were seeded on a petri dish (60-mm diameter; Becton-Dickinson Labware, Oxnard, Calif.) in DME medium with 30% fetal swine serum and incubated the indicated times. In the

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mitogen-stimulated cultures, 15×10^6 leukocytes were incubated in a test tube with 3 ml of DME medium with 30% fetal swine serum (12-ml culture tubes; Becton-Dickinson Labware) supplemented with 5×10^{-5} M 2-mercaptoethanol and the optimal concentrations of mitogen during 20 h. The cells were washed three times with serum-free DME medium and infected with ASF virus (MOI, 5 HADU/cell). After 4 h of adsorption, the cells were washed three times with DME medium and cultured in fresh DME medium with 30% fetal swine serum supplemented with 2-mercaptoethanol and, when indicated, mitogens. Cell cultures were incubated at 37°C in a humidified atmosphere of 7% CO₂ in air for 5 days, unless specified otherwise. For analysis, all of the cells in the culture were used by scraping in culture medium with a rubber policeman the attached cells, which were mixed with the unattached ones.

Antisera. Rabbit anti-swine thymocyte serum was kindly provided by D. H. Sachs (National Institutes of Health, Bethesda, MD.) or induced by sequential immunization with 6×10^8 swine thymocytes, first in Freund complete adjuvant (Difco Laboratories, Detroit, Mich.), second in Freund incomplete adjuvant (Difco), and then by five multisite immunizations with the same number of thymocytes administered at 1-month intervals. The rabbits were bled 7 to 10 days after the last injection. The serum was adsorbed by a modification of a previously described procedure (35). Briefly, the serum was incubated once (60 min) with 5 volumes of packed swine erythrocytes, twice with equal volumes of packed fresh swine bone marrow cells (overnight), twice with 1 volume of PK15 cells (3 h), and once with 1 volume of lyophilized normal swine serum (60 min). All of the adsorptions were performed at 4°C. Finally, the serum was centri-

fuged at $100,000 \times g$ (30 min). The adsorbed serum, at a 1:20 dilution, reacted to porcine thymocytes but not to erythrocytes, bone marrow cells, or macrophages in an indirect immunofluorescence test (data not shown).

Rabbit anti-swine immunoglobulin M serum was induced by immunization with a swine immunoglobulin M fraction prepared as described by Zikan et al. (36). The antiserum at a 1:20 dilution reacted only with 10% of porcine leukocytes (presumably B cells) but not with monocytes, macrophages, or thymocytes fractionated in nylon wool columns (16).

Mouse monoclonal antibodies specific for the ASF virus proteins vp73 (1B-C11) and vp12 (2C-G12) were produced and characterized as described elsewhere (L. Enjuanes, I. Casal, A. Sanz, B. G. Barreno, and E. Viñuela. 15th International Leucocyte Culture Conference. Immunobiology. 163:226, 1982; A. Sanz, B. García-Barreno, M. Nogal, E. Viñuela, and L. Enjuanes, manuscript in preparation) and used as a mixture (1:1) of hybridoma cell culture supernatants.

Swine antiserum specific for the porcine parvovirus (strain NADL-2) was kindly provided by P. S. Paul (National Animal Disease Center, Ames, Iowa) (22).

Immunoglobulins and F(ab)₂ fragments. Rabbit immunoglobulin G (IgG) was isolated by affinity chromatography on protein A-Sepharose CL-4B (Pharmacia) by previously described methods (18). F(ab)₂ fragments were prepared by peptic digestion of IgG and removal of the low-molecular-weight cleavage products by gel filtration (27) and residual IgG by chromatography on a protein A-Sepharose column. The F(ab)₂ fragments were free of IgG as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (data not shown).

FIG. 1. Susceptibility of porcine leukocytes to ASF virus. Leukocytes (20×10^6) were infected (MOI, 5 HADU/cell) with ASF virus BA71-5, and at the times indicated in the graphs, samples were examined to determine the production of infectious virus (A); the percentage of each cell type in the infected cultures, determined by indirect immunofluorescence (B^+ and T^+ , B and T lymphocytes, respectively) or the presence of esterase activity (M^+ , macrophages) (B); the percentage of infected cells with markers of B (B^+) or T (T^+) lymphocytes or without these markers (B^-T^-), determined by indirect double immunofluorescence (C); the percentage of each cell type (M^+ , macrophages; G^+ , PMNL; L^+ , lymphocytes) showing viral structures (D). Symbols: x, infectious virus; O, T lymphocytes; Δ , B lymphocytes; $\square$, non-T, non-B leukocytes; $\blacksquare$, lymphocytes; $\blacktriangle$, PMNL; $\bullet$, macrophages.

Characterization of cells. Leukocyte subsets were differentiated by three procedures: double immunofluorescence, cytochemical, and electron microscopy. (i) The double immunofluorescence staining procedure was adapted from a previously described method (13). Briefly, porcine leukocytes were washed with warm, serum-free DME medium for 30 min and once with PBS and were then stained for surface antigens in suspension. Surface immunoglobulin in B lymphocytes was detected with rabbit F(ab)₂ anti-porcine immunoglobulin M, and T cells were detected with rabbit anti-porcine thymocytes [identical results were obtained with the F(ab)₂ fragments of the last antiserum]. Cells from each tube were then washed and stained with rhodamine-labeled goat F(ab)₂ anti-rabbit IgG (Cappel Laboratories, Cochranville, Pa.) for 30 min at 4°C in the presence of 20 mM sodium azide. After washing, the cells were suspended at a concentration of 10⁶ cells per ml in PBS with 1% bovine serum albumin (Sigma Chemical Co.), and smears were prepared with a Cytospin 2 centrifuge (Shandon, London), fixed for 5 min in methanol-acetone 50% (vol/vol) at 4°C, and air dried. Each slide was treated with monoclonal antibodies specific for the ASF virus structural proteins vp73 and vp12 (30 min; 22°C), washed with PBS, and stained with fluorescein-labeled goat F(ab)₂ anti-mouse IgG (Cappel). After incubation (30 min; 22°C), the slides were washed three times with PBS and mounted in phosphate-buffered glycerol. Over 300 cells were examined on each slide under a Zeiss Photomicroscope II equipped with an epifluorescence device, III RS. (ii) Cells of the mononuclear phagocytic series were defined as those positive for the presence of nonspecific esterase (17). PMNL were characterized by the presence of chloroacetate esterase activity (34) and by May-Grünwald-Giemsa staining. (iii) Cell pellets for electron microscopy were fixed in 2.5% phosphate-buffered glutaraldehyde at 4°C (60 min), washed three times with PBS, postfixed on 2% osmium tetroxide (60 min), and washed again with PBS. Dehydration was accomplished by passing the specimens through a series of increasing acetone concentrations, and staining them with uranyl acetate. Vestopal (Serva, Heidelberg, Federal Republic of Germany) was used as embedding material (3). Ultrathin sections were made with a Sorvall ultramicrotome, stained with lead citrate (24), and examined in a JEOL 100B electron microscope.

RESULTS

Susceptibility of porcine leukocytes to ASF virus. Porcine leukocytes were infected with ASF virus (BA71-5) to determine at various times after infection (i) the production of infectious virus (Fig. 1A), (ii) the percentages of the various cell types (Fig. 1B), (iii) the percentages of infected cells with or without markers for B lymphocytes, T lymphocytes, or both (Fig. 1C), and (iv) the percentage of each cell type showing viral structures (Fig. 1D).

The relative number of T or B lymphocytes in the culture did not change significantly during the infection, in contrast to the fourfold decrease in the number of macrophages (Fig. 1B). In the same cultures, there was a virus production of ca. 5 HADU/cell (Fig. 1A). These results suggested that neither B nor T lymphocytes were infected by ASF virus. A critical test for lymphocyte sensitivity or resistance to ASF virus was to determine the presence or the absence of either viral antigens or virus-related structures in individual cells that contained T or B lymphocyte markers (Fig. 1C and 2) or showed a characteristic morphology under the electron microscope (Fig. 1D and 3). The results obtained indicated that most of the macrophages and ca. 4% of the PMNL were

FIG. 2. Double immunofluorescence labeling of ASF virus-infected leukocytes. Cells were stained with rabbit antibody to T⁺ or B⁺ cell markers plus rhodamine-labeled goat antibody to rabbit IgG and then with a mixture of two mouse monoclonal antibodies specific for ASF virus proteins plus a fluorescein-labeled goat anti-mouse IgG. A, A', B, and B', Leucocytes stained with antisera to ASF virus and to B-cell markers; A, fluorescein-excitation; A', rhodamine excitation of the field shown on A; B, fluorescein excitation; B', rhodamine excitation of the field shown on B. C, C', D, and D', Leucocytes stained with antisera to ASF virus and T-cell marker; C, fluorescein excitation; C', rhodamine excitation of the field shown in C; D, fluorescein excitation; D', rhodamine excitation of the field shown in D.

infected by ASF virus and that T and B lymphocytes did not contain either viral antigens or virus-related structures.

Infection of a subpopulation of PMNL by ASF virus. Figure 4 shows an analysis, similar to that indicated in Fig. 1, of a purified population of PMNL infected with ASF virus. The proportion of cells positive by indirect immunofluorescence for ASF virus antigens (Fig. 4C) was maximum (4.5%) at 66 h after infection when less than 0.5% of the cells in culture were esterase positive, indicating that the contaminant macrophages could not account for all antigen-positive cells. To verify these results, the cells were observed by electron microscopy at various times after infection. Figure 4D shows that the small percentage (ca. 0.5%) of contaminant macrophages, present at 40 h postinfection, were infected and that ca. 3% of the PMNL contained virus-related particles (Fig. 5C and D).

FIG. 3. Thin sections of (A) a virus factory, (B) lymphocytes, or (C) macrophages without virus-like structures and (D) a macrophage at early stages (16 h) after infection with ASF virus.

Resistance of mitogen-stimulated porcine lymphocytes to ASF virus. Some viruses (herpes simplex, vesicular stomatitis, measles, and rubella) can replicate in lymphocyte cultures *in vitro* when the cells are stimulated by mitogens (4, 6). To study whether the mitogenic stimulation of porcine lymphocytes made them susceptible to ASF virus, porcine leukocytes were incubated for 24 h with mitogens specific for T cells (PHA), B cells (LPS), and both (PWM). The mitogens were removed from the culture medium, and the cells were infected with ASF virus BA71-5 (MOI, 5 HADU/cell). After virus adsorption (4 h) and removal of the nonadsorbed virus, the cells were restimulated with the corresponding mitogen to study the replication of ASF virus in actively dividing lymphocytes. The infected cultures were analyzed as before to detect viral components in the leukocyte subpopulations. Figure 6 shows the results obtained for leukocytes stimulated with PHA and LPS. In both cases, the production of infectious virus was lower in the mitogen-stimulated than in the unstimulated leukocyte cultures (Fig. 6A and A'). Although the amount of infectious virus initially associated with the PHA-stimulated leukocytes was similar to the virus associated with the unstimulated ones, the production of ASF virus in the presence of PHA was inhibited by more than 95%. Figures 6B, B', C, and C' show

that the evolution of various cell types in the cultures with leukocytes stimulated with either mitogen was similar. By double immunofluorescence, neither B nor T cells expressed viral antigens after stimulation with PHA or LPS (Fig. 6C and C'). In addition, the number of B⁺T⁻ cells positive for viral antigens was 8 and 5% in the cultures stimulated with PHA and LPS, respectively. Almost all cells classified by electron microscopy as macrophages (100 and 95% in the cultures with leukocytes stimulated with PHA and LPS, respectively) contained virus-like structures but in lower numbers (specially in PHA-stimulated cultures) than in the cultures with unstimulated leukocytes. Among the cells classified as PMNL by electron microscopy (Fig. 6D and D'), ca. 2% contained virus-like structures in the cultures with leukocytes stimulated with PHA, and this percentage increased ca. fivefold in those stimulated with LPS. Again, neither B nor T cells contained virus-like structures in the leukocyte population stimulated with either PHA or LPS.

To study whether a simultaneous stimulation of B and T lymphocytes would make porcine lymphocytes susceptible to ASF virus, the leukocytes were incubated in the presence of PWM and infected with ASF virus. PWM-stimulated leukocytes incorporated two- to fivefold more [³H]thymidine than those stimulated with either PHA or LPS (data not

FIG. 4. Susceptibility of purified porcine PMNL ASF virus. Purified PMNL (>95%) were infected (MOI, 5 HADU/cell) with ASF virus BA71-5 and examined as described in the legend to Fig. 1. (A) Production of infectious virus; (B) percentage of each cell type in the infected cultures, determined by indirect immunofluorescence (L⁺, B and T lymphocytes), the presence of esterase activity (M⁺, macrophages), or May-Grünwald-Giemsa staining (G⁺, PMNL); (C) percentage of infected cells determined by indirect immunofluorescence; (D) percentages of each cell type (M⁺, macrophages; G⁺, PMNL; L⁺, lymphocytes) showing viral structures. Symbols: x, infectious virus; ▲, PMNL; ■, lymphocyte; ●, macrophages; □, total cells.

FIG. 5. Thin sections of purified PMNL 24 h after infection with ASF virus BA71-5. A, High magnification of a PMNL without ASF virus structures; B, low magnification of the PMNL culture; C, PMNL with ASF virus particles; D, high magnification of the region indicated in C.

FIG. 6. Resistance of PHA- or LPS-stimulated lymphocytes to ASF virus. Leukocytes (5×10^6 cells per ml) from 24-h mitogen-stimulated cultures were infected (MOI, 5 HADU/cell) with ASF virus BA71-5, and at the times indicated in the graphs, samples were examined to determine the production of infectious virus (A, A'); percentage of each cell type in the infected cultures, determined by indirect immunofluorescence (B^+ and T^+ , B and T lymphocytes, respectively), the presence of esterase activity (M^+ , macrophages), and May-Grünwald-Giemsa staining (C^+ , PMNL) (B, B'); percentage of infected cells with markers of B (B^+) or T (T^+) lymphocytes or without these markers (B^-T^-), determined by indirect double immunofluorescence (C, C'); percentage of each cell type (M^+ , macrophages; G^+ , PMNL; L^+ , lymphocytes) showing virus structures (D, D'). Symbols: x, virus titer; O, T lymphocytes; Δ , B lymphocytes; $\blacktriangle$, PMNL; $\bullet$, macrophages; $\square$, non-B, non-T leukocytes; $\blacksquare$, lymphocytes.

shown). Figure 7 shows that the production of infectious virus was inhibited by more than 96% in relation to the production of virus by unstimulated leukocytes (Fig. 7A). The evolution of the various cell types is shown in Fig. 7B. By double immunofluorescence, neither B^+ nor T^+ lymphocytes expressed viral antigens (Fig. 7C), and the percentage of B^-T^- cells with viral antigens was about 16-fold lower than that observed in the unstimulated cultures at 42 h postinoculation (Fig. 1C). By electron microscopy, larger numbers of lymphoblasts and plasma cells were observed in

the stimulated leukocytes than in the unstimulated ones, although the number of cells with virus-like structures was very small (data not shown), in agreement with the inhibition of infectious virus production and the results of the double immunofluorescence shown above.

The inability of porcine B and T cells to replicate ASF virus could be due to inactivation during handling of the cells. To study this possibility, PHA-stimulated porcine leukocytes, treated as indicated in Fig. 6, were infected with a porcine parvovirus able to replicate in stimulated lympho-

FIG. 7. Resistance of PWM-stimulated lymphocytes to ASF virus. Leukocytes (5×10^6 cells per ml) from 24-h mitogen-stimulated cultures were infected (MOI, 5 HADU/cell) with ASF virus BA71-5, and at the times indicated in the graphs, samples were examined to determine the production of infectious virus (A); percentage of each cell type in the infected cultures, determined by indirect immunofluorescence (B^+ and T^+ , B and T lymphocytes), the presence of esterase activity (M^+ , macrophages), or May-Grünwald-Giemsa staining (G^+ , PMNL) (B); the percentage of infected cells with markers for B (B^+) or T (T^+) lymphocytes or without these markers (B^-T^-), determined by indirect double immunofluorescence (C). Symbols are as in the legend to Fig. 6.

cytes (22). The cultures were screened by double immunofluorescence to determine the infected cell types. The results obtained indicated that at 48 h postinfection, ca. 5% of the cells contained porcine parvovirus antigen, and from them 3.5% were T cells, suggesting that the inability of porcine T or B cells to replicate ASF virus was not due to inactivation of the cells during handling.

Resistance of PHA-stimulated porcine leukocytes to highly passaged ASF virus. The experiments described above were

performed with a clone of ASF virus (BA71-5) passed five times in leukocyte cultures. To study whether the continuous passage of ASF virus in porcine leukocytes would select a variant virus able to infect porcine B and T lymphocytes, we carried out experiments similar to the ones described in the legend to Fig. 6 with a virus isolate that has been passaged 100 times in leukocyte cultures (BA71-100). Figure 8 shows that ASF virus BA71-100 replicated on unstimulated porcine leukocytes to about the same extent as BA71-5 and that the production of highly passaged ASF virus was also inhibited by PHA (Fig. 8A). Although T cells were clearly stimulated by the mitogen and their relative percentage increased from 35 to 58% (at 30 and 40 h postinfection, respectively) (Fig. 8B), neither T nor B lymphocytes were positive for viral antigens by double immunofluorescence (Fig. 8C). In contrast, ca. 2% of B^-T^- cells contained viral antigens. These results were confirmed by electron microscopy (Fig. 8D). At 30 h postinfection, ca. 50% of the cells classified as macrophages, which represented only ca. 2% of the total cell number, and 3 to 4% of the PMNL contained virus-like structures (most of them were empty virus capsids). In contrast, no cell with lymphocyte morphology contained virus-like particles.

Figure 9 shows that the inhibition of ASF virus replication in mitogen-stimulated leukocytes (Fig. 6 to 8) was not due to a blocking of the virus receptor by the mitogen, since the extent of the inhibition was about the same when the lymphocytes were stimulated before, after, or before and after virus adsorption.

DISCUSSION

In this study, we have shown that ASF virus replicates *in vitro* in most, if not all, porcine macrophages from peripheral blood leukocytes and in a small subset (ca. 4%) of the PMNL but not in B or T lymphocytes. The susceptibility of a subset of PMNL to ASF virus can be understood on the basis that both cell types have a common precursor (20). As the percentage of infected PMNL was small, it could be possible that some infected macrophages (the other cell type susceptible to ASF virus infection in porcine leukocytes) have been erroneously classified as PMNL. This possibility seems unlikely, because when the susceptibility to ASF virus of a purified population (>96%) of PMNL was studied, ca. 4% of the PMNL were susceptible to the virus when less than 0.5% of the cells present were esterase positive.

B and T lymphocytes were resistant to ASF virus infection even after mitogenic stimulation with PHA, LPS, or PWM. The lack of susceptibility to ASF virus of stimulated B and T cell differs from other viral systems (herpes simplex, vesicular stomatitis, measles, and rubella virus) (4, 6) in which the mitogenic stimulation of the lymphocytes makes them susceptible to viral infection. The possibility that the mitogen could block the viral receptors on B or T cells was ruled out in experiments in which the mitogen was added 4 h after the virus infection. In addition, it is very unlikely that all of the mitogens (PHA, LPS, and PWM) used to stimulate the lymphocytes would block the same viral receptor. The possibility that the mitogens inactivated the virus infectivity was discarded, since ASF virus was stable for at least 4 days in the presence of the mitogen concentration used for the stimulation under the conditions (37°C) of the experiments described above. The possibility of lymphocyte inactivation during the handling was unlikely because, at least, PHA-stimulated T cells from porcine leukocytes isolated in a similar way were able to replicate a porcine parvovirus.

FIG. 8. Resistance of PHA-stimulated porcine lymphocytes to highly passaged ASF virus. Leukocytes from 24-h mitogen-stimulated cultures were infected (MOI, 5 HADU/cell) with ASF virus BA71-100 and analyzed as described in the legend to Fig. 5. (A) Production of infectious virus; (B) percentage of each cell type in the infected cultures, determined by indirect immunofluorescence (B⁺ and T⁺, B and T lymphocytes, respectively), the presence of esterase activity (M⁺, macrophages), or May-Grünwald-Giemsa staining (G⁺, PMNL); (C) percentage of infected cells with markers of B (B⁺) or T (T⁺) lymphocytes or without these markers (B⁻T⁻), determined by indirect double immunofluorescence; (D) percentage of each cell type (M⁺, macrophages; G⁺, PMNL; L⁺, lymphocytes) showing viral structures. Symbols are as in the legend to Fig. 6.

If ASF virus could replicate on B or T lymphocytes after mitogenic stimulation, an increase in the production of virus after the stimulation of the infected leukocyte cultures could be predicted. In contrast, inhibition of infectious virus

production was observed. This inhibition could be due to a positive mechanism mediated by a soluble factor like interferon (32) or to a negative mechanism, such as an increase in the arginase level, that may deplete the medium of an

FIG. 9. Effect of the PWM stimulation of porcine leukocytes, before and after ASF virus infection, on the production of infectious virus. Porcine leukocytes were incubated for 24 h in the absence or presence of PWM, infected (MOI, 5 HADU/cell) with ASF virus BA71-5 for 4 h, and further incubated in the absence or presence of PWM; the production of infectious virus was determined. Symbols: ●, cells stimulated with PWM before and after virus inoculation; ▲, cells stimulated only before virus infection; ■, cells stimulated only after virus infection; ○, unstimulated cells.

essential nutrient (33). The production of interferon by mitogen-activated macrophages or T cells as a possible mechanism for ASF virus inhibition would be in agreement with observations done with other viruses (2, 12, 14, 32). The possibility of an induction of arginase in stimulated macrophages has been suggested previously (33) as the mechanism of inhibition of herpes simplex virus replication by activated macrophages. Maximum inhibition of virus replication (98%) was achieved when the mitogen was present before and after virus infection. Similar results (not shown) were obtained when purified macrophages were cultured in the presence of PWM, suggesting that this mitogen inhibited virus production through a direct action on the macrophage or, alternatively, induced in the cell a virus-inhibiting factor.

In previous studies on the susceptibility of porcine lymphocytes to ASF virus infection, some authors have concluded that the lymphocytes were infected by the virus (31) and that the cytolytic infection of lymphocytes affected mainly B cells (30). In contrast, other authors have concluded that the virus affects T lymphocytes and monocytes in culture to a much greater degree than to B lymphocytes and neutrophils (26). The observed differences were tentatively assigned to the use of different virus strains and to the number of passages of the virus in cell culture (26, 29). In addition, the different behavior of the virus also could be due to the virulence of the particular viral isolate used. It is unlikely that the use of different virus strains explains the different results obtained, since we have obtained similar results with porcine T and B cells infected with viruses passaged 5 (BA71-5) or 100 times (BA71-100). Furthermore, for the two ASF virus clones used, one was of low virulence and the other was of high virulence (C. Mebus, personal communication). These results indicate that the lack of susceptibility to ASF virus or porcine B and T cells probably is not due to differences in virus passage number or to the virulence of the particular virus isolate.

The fact that most porcine macrophages and ca. 4% of PMNL (which, in absolute terms, is a number close to the number of macrophages in peripheral blood) are infected by ASF virus may have important implications in the immune response to the virus. The importance of the macrophage in herpes simplex, lactate dehydrogenase, equine infectious anemia, and other virus infections has been clearly established (1). The hypergammaglobulinemia induced by ASF virus in chronically infected animals (21) could be due to an unusual increase in the amount of viral antigen available in the surface of the antigen-presenting cell, the macrophage, due to the capability of this cell to multiply the internalized antigen. An alternative explanation, the susceptibility of suppressor T cells to the virus, seems unlikely since the T lymphocytes do not seem to be susceptible to ASF virus.

Although we have shown that porcine B and T cells are not infected *in vitro*, it will be important to study whether these cell subsets are also resistant to ASF virus *in vivo* when other conditions may be operating. Experiments to clarify this point are in progress.

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