

MINISTÉRIO DA AGRICULTURA, FLORESTAS E ALIMENTAÇÃO
DIRECÇÃO-GERAL DA PECUÁRIA
LABORATÓRIO NACIONAL DE INVESTIGAÇÃO VETERINÁRIA

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PROGRESS REPORT

Project No.: 936-5542
Grant No.: DPE-5542-G-SS-7041-00
Project office: S&T/AGR
Obligation No.: 7361133

Date: February 20, 1988

INTRODUCTION

In this document we report the work that has been developed from September 87 to the present, to pursue the objectives described on PHASE I and II of the above mentioned project.

During this period, work was developed to set up the basic experimental conditions necessary for the execution of the project:

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- i) Preparation of general culture media and reagents,
- ii) Maintenance of L929 (mouse fibroblast) cell line and preparation of crude macrophage growth factor CSF-1 (L929 conditioning media).
- iii) Preparation of porcine blood leukocyte (PBL) cultures.
- iv) Preparation of enriched cultures of porcine blood derived macrophages.
- v) Propagation and production of ASFV/NH/P68 working suspensions.
- vi) Viral titration.
- vii) Preliminary evaluation of pathogenic and immunogenic characteristics of ASFV/NH/P68 in pigs inoculated by IM route.

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MATERIALS AND METHODS

Animals

Conventionally raised pigs 3 to 4 months old, from LNIV's animal colony were used as blood donors for the culture of peripheral blood derived macrophages and for the *in vivo* studies with ASF/NHV/P68 .

Virus

Swine peripheral blood collected at day 4 PI with a naturally occurring nonhemadorbing ASF virus isolated in Portugal in 1968 (Vigário et al 1974), designated ASFV/NH/P68 was used as source of working virus.

Production of Macrophage Growth Factor CSF-1 (L929 conditioning medium):

To stimulate porcine macrophage growth, a crude growth factor was prepared as previously described from the supernatants of mouse fibroblast L-929 (ATCC #CCL 1) cultures



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(Burgess et al. 1977, Stanley and Heard 1977, and Warren and Ralph 1986). Briefly, L-929 cultures were maintained in 25 cm² tissue culture flasks (Costar) and in RPMI supplemented with 10% FCS at 37°C, >90% Humidity, in 5% CO₂ in air. The cells were trypsinized and subsequently passaged to T75 TC bottles (Costar) and glass Roux flasks. After the monolayers became confluent in these flasks, cells were further incubated for about one week. Then culture media was collected and centrifuge clarified at 600g, 4c, for 45 min. The media was pooled, filtered through .22um (Millipore Corporation, Bedford, MA), equated and stored at -20°C until use. This crude L929 conditioned media (CM) has been shown by others to promote the proliferation of porcine mononuclear phagocytes (macrophages) susceptible to ASFV (Pan I 1987).

Porcine peripheral blood leukocyte (PBL) cultures:

PBL's were obtained by our routine procedure. Briefly, blood was collected from the anterior vena cava of animal donors and coagulation prevented by heparin(10 U.I./ml blood).The blood was then mixed thoroughly with 10% (v/v) of a 5% Dextran (Dextran T 500, Pharmacia Chemicals) solution in Hanks BSS in 200 ml Erlmeyer flasks and incubated at 37°C for 10 to 15 minutes in a tilted position, allowing the sedimentation of the majority



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of erythrocytes. The leucocyte rich supernatant plasma (SP) was removed and layered over Ficoll - Hapaque ($d=1.077$, Pharmacia Fine Chemicals) in a proportion of 2:3 (F/H:SP) in conical centrifuge 50 ml tubes and centrifuged for 30 minutes at 400g at room temperature. The interface cell layer was collected, suspended and washed 3 times in Hanks BSS at 200 g for 10 minutes at 4°C. The supernatant obtained after the leukocyte collection over Ficoll Hapaque was cleared by centrifugation at 600 g for 30 minutes at 4°C and used as autologous plasma supplement (AP) in the culture medium. After the final wash, PBL were resplendent in complete culture medium RPMI 1640 (Flow Laboratories, McLean Va.) supplemented with 30% of autologous plasma (AP), 20% L929 Conditioning medium, 10% Foetal Calf serum, 200mM L-Glutamine, 100 I.U. penicillin/ml and 100 g streptomycin/ml and buffered with 25mM Hepes. Cell viability assessed by tripan blue dye exclusion was always superior to 90%.

Cells in suspension in culture medium, were seeded in T75 TC glass bottles (200×10^6 cells/flask) and incubated at 37°C in a 5% CO₂ atmosphere and >80% relative humidity.

Porcine-macrophage enriched cultures:

PBL's obtained as above, were incubated for 72 hrs. Then,



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flasks were washed 3x in warm phosphate buffered saline (PBS). Cells were detached after incubation for 5 mins at 25°C with a 1:3000 Disodium Ethylenediamine Tetraacetate (EDTA) solution in PBS and were resuspended in RPMI supplemented with 10% FCS at a concentration of 1×10^6 cells/ml. Cell suspension was distributed into 96 well flat bottomed microtiter plates (Costar, Cambridge, MA) - 100ul/well. The plates were centrifuged at 200g for 4 mins, 25c and incubated at 37°C for 3-4 hours.

Production of (ASF/NH/P68) working suspension

To produce viral suspensions, we have used the PBL culture system described above. PBL were prepared in T75 glass bottles, containing 200×10^6 cells in 30ml of media and maintained loosely stopped at 37c, >90% humidity, in 5% CO2 in air. Within 4-6 days, a near confluent monolayer of adherent cells was obtained. Then, culture medium was decanted, cells rinsed with warm HBSS and inoculated with 10ml of stock virus. After one hour incubation at 37°C, supernatant was decanted, cells washed and medium culture added. Cell cultures were daily monitored for CPE and flasks were frozen at -70c when 50-75% of the adherent cells were in suspension (72-96 hr post-innoculation). After complete freezing the flasks were



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slowly thawed at 25c. The virus suspension was then pooled and clarified by centrifugation at 800g, 4°C for 20 mins. Following centrifugation, the supernatant was decanted, equated and stored at -70°C until use.

Virus titration

The virus was titrated by observing its cytopathic effect (CPE) in endpoint dilution on enriched porcine macrophage cultures as described above.

The viral suspension was 10-fold serially diluted in RPMI with 10% FCS; 100ul of each dilution was added to 5 replicate wells. The wells were scored daily for the presence of CPE for one week. The viral titer was calculated using Spearman-Kärber analysis and expressed as 50% CPE doses (CPE50).

Inoculation of pigs

Recently, 3 groups of four pigs weighing 15-20 kg each, were inoculated with ASF/NHV/P68 by IM route :

Group I - four pigs inoculated with 2×10^6 CPE50.

Group II - four pigs inoculated with 2×10^5 CPE50.



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Group III - four pigs inoculated with 2×10^3 CPES0.

Studies on pigs experimentally inoculated:

The effect of viral infection in the inoculated animals has been recorded as follows:

Daily - observation of clinical signs and body temperature.

Twice a week -- blood sampling. Blood samples are used to study:

Hemogram,

Virus isolation and titration,

Viral and lectin (PHA, Con A and PWM) induced blastogenesis

Viral specific antibodies and antigen(s) kinetics.

General comments:

So far, the work developed during the first period related to the project execution, has been carried out within the timing



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previously planned.

Although, the acquisition of some equipment has been delayed by the need to reach a balance between the suppliers payment conditions and the prompt availability of funds from our institutions.

J. P. Matos Aguas
Director do L.N.I.V.

J. Dias Vigário

Principal Investigator



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FINANCIAL STATUS REPORT

Due to the fact that the purchase of equipment, coming from abroad (U.S. in our case) and the formalities to which our Institutions are obliged by the Portuguese State rules concerning the supply of equipments and admission of personal, we are only able to send you our voucher for cost reimbursement concerning the first months of execution of our project.

We would also inform you that the salary of Miss Margarida Duarte has been paid in advance by LNIV only for the period concerning 20 August to 30 September 1987 and since then (October 1987) she has been paid by a National Fellowship granted by the JNICT (Lisbon). This situation was the best issue let to us to solve the problems arisen by recent determinations related to personal engagement. By this reason we are thinking to transfer the remaining amount of this salary to cover our needs for materials and reagents necessary for the project (First year salary U.S. \$5300.00 - \$630.00 = \$4670.00 to be transferred to direct costs).

All the equipment acquired has been delivered during the last 30 days as confirmed by the attached documents from the suppliers.

In the following table we summarize the expenditure made from September 87, to which are available the suppliers receipts at the present time.



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BALANCE (August 1987 - February 1987)

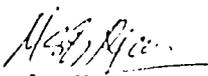
CURRENCY: PORTUGUESE ESCUDOS. The figures in parentheses refer to the first year expenditure provisions expressed in U.S. Dollars.

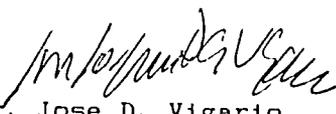
	LNIV	ESMV	TOTAL
Direct Salaries	87,362 (\$5,300)	334,304 "" (\$4,565)	421,666
Equipment	- (\$40,000)	1556,300 (\$11,590)	1556,300
Materials & Reagent	394,203 (\$2,500)	- (\$3,335)	394,203
Training	- (\$18,000)	- -	-
Travel	- (\$3,000)	- (\$5,000)	-

TOTAL IN PORT. ESCUDOS:

Two millions three hundred seventy two
thousands one hundred sixty nine escudos 2372,169

""Salaries of Mrs Clara Cartaxeiro from August 21, 1987 to
February 29, 1988.


Dr. J. Matos Aguas
Director LNIV


Dr. Jose D. Vigarrio
Principal Investigator