VIRAL IMMUNOLOGY
Volume 4, Number 1, 1991
Mary Ann Liebert, Inc., Publishers

Juli's Coll

6

Stimulation of Adherent Cells by Addition of Purified Proteins of Viral Hemorrhagic Septicemia Virus to Trout Kidney Cell Cultures

A. ESTEPA, B. BASURCO, F. SANZ, and J.M. COLL

ABSTRACT

Purified proteins of the virus causing viral hemorrhagic septicemia in the trout were added to cultures on semisolid medium of leukocytes obtained from either healthy or immunized rainbow trout. Adherent cells were specifically stimulated by the glycoprotein of the viral spikes and, to a lesser extent, by the nucleoproteins. In contrast, a specific memory response was associated more with the nucleoproteins than with the glycoprotein when leukocytes from trout immunized with the virus were employed. These results suggest the necessity of employing both proteins in subunit vaccination trials and the possibility of using this assay to select the proper epitopes for genetically engineered proteins during subunit vaccine development.

One of the greatest drawbacks in the development of subunit viral vaccines is that we do not know enough about the antiviral defensive mechanisms of mammals. Despite numerous investigations on the complex issues of cellular immunology (6,11) and viral structure and biology, nonreplicating subunit vaccines continue to be a nonpractical technology in need of further studies (4).

Because of the complexity of the mammalian models, we choose an evolutionarily more primitive animal, the trout, to study immunologic responses to a well-known pathogen, the rhabdovirus causing viral hemorrhagic septicemia (VHS) in salmonids. On one hand, fish are of the most primitive animals to possess an adaptative immune system characterized by the existence of cells (not yet demonstrated to possess B and T lymphocyte cells) and only one class (IgM-like) immunoglobulins (18). It is therefore of interest to study how their immune cells react against viral antigens. On the other hand, the virus chosen for this study is not only a well-known rhabdovirus but also the cause of 30% to 50% of the annual losses in the European production of trout.

The VHS virus has five proteins: a glycoprotein (G; 65 kD), two membrane proteins (M_1 and M_2; 24 and 20 kD, respectively), a large-minority protein (L; 200 kD), a phosphorylated nucleoprotein (N; 38 kD) associated

Instituto Nacional de Investigaciones Agrarias, Centro de Investigatcion y Tecnologia, Departmento de Sanidad Animal, Madrid, Spain

with the virion, and a nucleoprotein (Nx; 34 kD) specifically associated with incomplete nucleocapsids (manuscript in preparation). The internal proteins—the L, N, and Nx—are complexed with the viral RNA to form the viral ribonucleoprotein. The external protein, G is the only antigen able to induce a virus-neutralizing response in trout (3,12,14).

To study the response of trout leukocytes, we developed an *in vitro* fibrin-clot system in which mammalian lymphocyte mitogens induce the proliferation of five morphologic types of cells. Lymphocytes, large-nucleated cells, and eccentric-nuclei cells, multinucleated cells, and adherent cells are stimulated by different mitogens used (phytohemagglutinin, concanavalin A, or LPS). In this system, none of these cells support the replication of the infectious pancreatic necrosis (IPN) virus (another virus of salmonids), whereas all of them are lysed by the VHS virus (manuscript in preparation).

In this report, we describe the use of purified viral proteins as stimulators of leukocytes obtained from either healthy or immunized trout. The results suggest that the G protein stimulated adherent cells from any trout, whereas the N and the Nx proteins stimulated adherent cells from primed rather than from healthy trout.

MATERIALS AND METHODS

Virus purification. The VHS virus was isolated in Spain from the rainbow trout *Salmo gairdneri*. The virus was grown in epithelial papullosum cyprine cells (EPC) as described before (1,2). Supernatant fluids from infected EPC cultures were centrifuged at 3000× *g* for up to 30 min and the pellet discarded. The supernatant fluid was made up to 7% polyethyleneglycol (PEG-6000) and 2.3% NaCl and agitated at 4°C for 2 h. After centrifugation at 10,000× *g* for 45 min, the pellet was resuspended in a small volume of TNE (0.15 M Tris, 0.15 M NaCl, and 1 mM EDTA pH 7.6). Analysis of this fraction by gel electrophoresis (13) showed it to be 90% viral proteins. This fraction will be called concentrated virus. The PEG-concentrated virus was layered on a 15% to 45% sucrose gradient in TNE and spun at 80,000× *g* for 270 min in a Beckman 25–75 rotor SW27 as described before (2).

Two bands were collected, at 31% sucrose and 26% sucrose. The band at 26% sucrose contained the viral infectivity (purified virus). Analysis of this fraction by gel electrophoresis showed it to be 98% viral proteins L, G, N, M_1, and M_2. The band at 31% sucrose contained free nucleocapsids by electron microscopy, and analysis of this fraction by gel electrophoresis showed it to contain 90% of viral proteins N and Nx (manuscript in preparation). This fraction will be called nucleocapsids.

Purified virus and supernatant fluid from VHS virus-infected cell monolayers were heated at 100°C for 10 min to heatkill the virus. Viruses were titrated by the $TCID_{50}$ method as described (1).

Purified virus was treated with 1% Triton ×100 of 1% Tween 20 during 30 min at room temperature and centrifuged over a 20% sucrose cushion at 80,000× *g* for 3 h.

By densitometry of the Coomassie blue-stained bands after electrophoresis, the viral particles isolated in the above-described way contained the viral proteins L, N, M_1, and M_2. The G protein was lost from these particles. These particles will be called Triton-treated and Tween-treated virus.

Purification of viral proteins. The VHS viral proteins were isolated by preparative gel electrophoresis of purified VHS virus in the presence of SDS and after denaturation by SDS and mercaptoethanol (13). Preparative electrophoresis was performed in a 15% polyacrylamide separating gel with a 6% staking gel. After electrophoresis, the gel edges were stained with Coomassie blue and the unstained bands between the edges cut at approximately the following molecular weights: 60–80 kD for G, 40–45 kD for N, 30–40 kD for Nx, 20–25 kD for M_1, and 15–20 kD for M_2. The bands were cut in small pieces and transferred to a dialysis bag containing 2 ml of 0.2 M Tris/sodium acetate pH 7.4 with 1% SDS. The proteins were electroeluted in 0.05 M Tris/sodium acetate pH 7.4 with 0.1% SDS at 100 V 100 mA for 3 h. After electroelution, the bags were dialyzed against 0.2 M ammonium bicarbonate. The protein solution was extracted from the bags with a Pasteur pipette, lyophilyzed, and resuspended in 50 μl of distilled water. Precipitates were discarded after centrifugation.

Isolated proteins were reelectrophoresed, and the gels were stained by silver nitrate (Biorad Silver Staining Kit, Richmond, VA). Protein content was first estimated by absorbance at 280 nm using ϵ of 1.4 and that value used to calibrate the silver nitrate staining estimate. The protein content was adjusted to 200–300 μg/ml, and proteins were kept frozen at −20°C until used.

Confirmation of identity of isolated viral proteins by using monoclonal antibodies. Female BALB/c mice were given nine intraperitoneal injections of 25 μg of concentrated VHS viral protein over a period of 9 months. During immunization, antibodies against VHS virus were monitored by indirect ELISA and immunoblotting. Spleen cells from the immunized mice were fused with the myeloma cell line P3-X63-Ag8653 as described (15). Fusion, cloning twice by limiting dilution, and cultivation of hybridoma cells and ascites production were performed as described previously (17,19).

The assay used for the screening of the hybridoma supernatant fluids was essentially as described (15). Briefly, microtiter plates (Dynatech, Plochingen, Germany) were coated to dryness with 0.5 μg of concentrated virus, washed for 15 min, and kept dried for weeks. Plates coated with concentrated EPC extracts were used as controls. Horseradish peroxidase-conjugated rabbit immunoglobulin against mouse immunoglobulins (Nordic, Tilburg, The Netherlands) was used to develop the reaction between hybridoma supernatant fluids and the virus-coated solid phase. An ascites pool from immunized mice was used for the positive control (dilution of 1000-fold). Development with *o*-phenylenediamine was by using the low background buffer as described previously (15). A well was considered positive when absorbance at 492 nm in the virus-coated wells was above 0.3 of that in the well coated with the noninfected cells.

The protein bands of a concentrated VHS virus were transferred from 12% polyacrylamide gels to nitrocellulose membranes. After blocking with dilution buffer (0.5% bovine albumin, 0.1% Tween-20, 0.01% Merthiolate, 5 mg of phenol red/L in 10 mM sodium phosphate, 0.15 M sodium chloride; pH 7.6), the strips were incubated 1 h with the hybridoma fluid diluted 1:2 in dilution buffer. The bands were developed with diaminobenzidine 1 mg/ml in citrate buffer as described above.

Confirmation of the identity of the isolated viral proteins was made by enzyme immunoassay. Isolated viral protein (1 μg) was bound per well of 96-well plates (Dynatech) and reacted with the monoclonal antibodies (Mabs) against the VHS virus following the procedures described above. Positive reactions showed values 5 to 10-fold higher than background.

Immunization of trout. Trout (5–20 g body weight) were purchased from commercial farms after several annual tests indicated that the fish were free of infectious pancreatic necrosis. The trout were held in 30-L aquaria with dechlorinated free-flowing water at 12°–18°C until used. Control trout were injected intraperitoneally twice during 2 months with phosphate-buffered saline diluted 1:1 with complete and incomplete Freund's adjuvant (200 μl of total volume per trout per injection). The VHS-positive trout were injected intraperitoneally 4 times during 3 months with 30 μg of killed PEG-concentrated VHS virus diluted 1:1 with complete Freund's adjuvant during the first injection; the remaining injections were diluted 1:1 with incomplete Freund's (200 μl of total volume per trout per injection).

Cells from pronephros. The trout were chilled to 4°C and bled by the tail vein to reduce the blood content of the kidney. The anterior kidney (pronephros) was removed under sterile conditions using a laminar flow cabinet and sterilized materials and cut into pieces with scissors in a Petri dish. Clumps of cells were dissociated by passing the suspension through a 20-gauge needle. The cell suspension was decanted briefly from the undissociated tissue, centrifuged at 1000×*g* for 10 min, and washed twice in cold cell culture medium. The cell concentration was determined with a hemocytometer and adjusted to 240,000 round cells/ml. Mature erythrocytes, distinguished by their oval shape, were not included in the counts. The contamination with mature erythrocytes was less than 10%. Cell viability, as determined by trypan blue exclusion, was higher than 80%.

Cell culture in fibrin clots. The cell culture medium was RPMI-1640 Dutch modification (Flow Lab, Ayrshire, Scotland) with 2 mM L-glutamine, 1 mM sodium pyruvate, amphotericin 1.2 μg/ml, gentamicin 50 μg/ml, 20 mM HEPES, 50 μM mercaptoethanol, 10% pretested fetal bovine serum, and 0.5% pooled rainbow trout serum. Fibrinogen (A.B. Kabi, Stockholm) was added to the medium just before use to a final concentration of 0.2 mg/ml. Thrombin (Miles Laboratories, Elkhart, IN) was added to the wells to a final concentration of 2–4 NIH U/ml.

Cultures of leukocytes were always from individual fish to avoid any possible mixed lymphocyte-type reactions. The cell suspension was prepared, thrombin was added at 0.2–0.4 NIH U/well in 2-μl volume, and 100 μl of the cell suspension was pipetted into each well of a 96-well plate (Costar; The Netherlands). After the clotting (in about 30 sec), the viral proteins diluted in sterile water were pipetted into each well in a maximum volume of 10 μl on the top of the clot. The plates were then sealed in a 20 × 12-cm plastic bag

(Vaessen; Schoemaker Indtal, S.A., Sant Bio de Llobregat, Barcelona, Spain), gassed with 5% CO_2 in air, sealed, and incubated at 20°C for 2 weeks.

After the incubation, the fibrin clots were removed from the wells onto a glass slide. The clots were partially dehydrated by placing filter paper on their surfaces. Then, 1.7% glutaraldehyde in 0.1% M sodium phosphate, 0.15 M sodium chloride, pH 7.4 was added for 10 min. The papers were removed and the clots washed in water and air dried. Staining was for 10 min in a 0.01 M sodium borate solution with 0.025% toluidine blue and 10 min in water. The preparations were mounted with Permount (Fisher Scientific). Other details were as described before (17).

Infection of the fibrin-clot cultures. To challenge the leukocyte cultures with live virus, pH conditions were chosen so that virus infection proceeded slowly (pH 7.2–7.4). Trout kidney cells were cultured with the viral proteins for 1 week at 20°C; then half of the cultures were infected with VHS virus, 10^5 PFU in 10 μl per well (10^6 PFU/ml), sealed, gassed with 5% CO_2 in air, incubated for 1 more week at 14°C and finally fixed and processed as described above. The virus challenge used will be called mild.

RESULTS

ELISA analysis of purified VHS viral proteins with MAbs. Following electrophoresis-electroelution of the proteins, their reactivity with Mabs was studied by ELISA. Four Mabs against the N and the Nx protein, two against M_2, one against M_1, and two against G were shown to recognize the respective electroeluted proteins by ELISA (Table 1 and data not shown). Reelectrophoresing and silver nitrate staining of electroeluted proteins indicated more than 90% purity for G, M_1, and M_2; N was 10% contaminated with Nx, and Nx was 27% contaminated with N (not shown).

Addition of viral proteins to leukocytes from healthy trout. Most of the pronephros cells remaining in the culture after 2 weeks belonged to the adherent cell type. Their number ranged from 250 to 500 per well, depending on the experiment. Their morphology is shown in Figure 1.

The addition of purified heat-killed VHS virus (5 μg of protein/ml) to the cultures increased the number of adherent cells about two-fold (Fig. 2). The addition of 15 μg of protein G/ml not only increased the number of adherent cells about 10-fold but also increased their average diameter from 30 ± 12 μm to 54 ± 20 μm (n = 30). Increasing the concentration of G to 30μg/ml did not increase these effects. The addition of 2.5 μg of either N or Nx per ml increased the number of adherent cells about fourfold, but larger amounts of these proteins were inhibitory. The addition of M_1 or M_2 proteins or purified killed IPN virus did not increase the number of adherent cells with respect to the controls.

Control trout were injected with adjuvant alone, and the leukocytes from the pronephros were cultured with

TABLE 1. RECOGNITION OF ELECTROELUTED VHS VIRAL PROTEINS BY ELISA USING MABS TO THE VHS VIRAL PROTEINS

Clone number	*Isotype*[a]	*Blotting*	*Neutralization*	*Electroeluted proteins*				
				G	*N*	*Mx*	*M1*	*M2*
1H10	IgG_1 K	G	+	+	−	−	−	−
2C9	IgG_{2a} K	N, Nx	−	−	+	+	±	±
1C10	IgG_1 K	M_1	−	−	−	−	+	−
4E4	IgM_1 K	M_2	−	−	−	−	−	+
Polyclonal	-	G, N, Nx, M_1, M_2	+	+	+	+	+	+

[a]Isotype was determined by ELISA with a Bioradkit. The Mabs were selected by ELISA on concentrated VHS virus-coated wells as described in Materials and Methods. Neutralization was performed as described (1, 2). Electroeluted proteins were used to coat the solid phase at 1 μg of protein/well. Mabs were 10-fold diluted and allowed to react with the solid-phase electroeluted proteins. Rabbit anti-mouse Ig μg of G labeled with peroxidase was used to detect the reaction. The results of ELISA were classified as positive (+) when the A492 nm was at least 0.3 in the protein-coated wells after subtracting the background; otherwise, they were considered negative or doubtful (±).

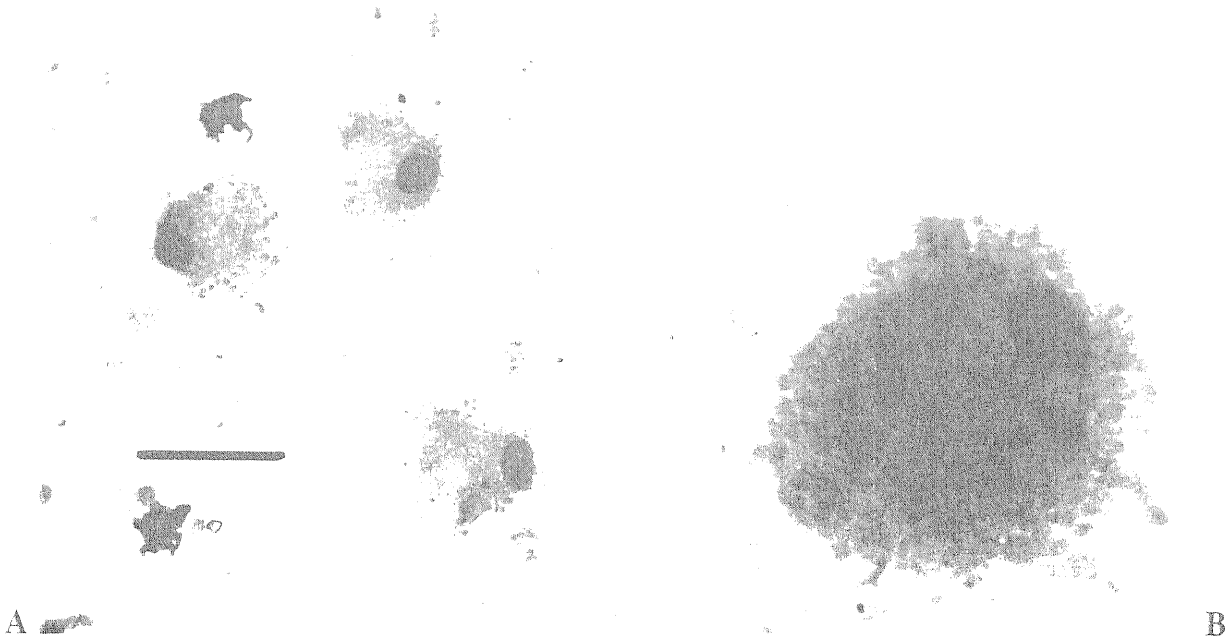

FIG. 1. Morphology of adherent cells stimulated by addition of isolated viral proteins. Adherent cells were predominant in cultures made in the presence of 15 μg of G/ml. They were scattered throughout the clot and very seldom formed colonies. The bar is about 30 μm. Photograph B is at 2.5-fold higher magnification than A.

treated virus and with isolated viral proteins. Cells appearing in the clots were classified as multinucleated, eccentric-nuclei, or adherent. In the control trout, the multinucleated cells were not abundant. Their number increased about 10-fold when Tween-treated virus was added to the cultures (Fig. 3). However, these stimulated cells were killed by mild virus challenge. The eccentric-nuclei cells increased from about 300/well to 1000/well in the presence of Triton or Tween-treated virus, nucleocapsids (Fig. 3), and N protein (Fig. 4). They seem to be resistant to mild virus challenge. The adherent cells increased from about 200/well to 2000 (Tween-treated virus) or 4000 (G and N proteins) per well. All these cells seemed to be resistant to the mild virus challenge (Figs. 3 and 4).

Addition of treated virus and viral proteins to leukocytes from immunized trout. In immunized trout, multinucleated cells were not stimulated by isolated purified viral proteins, but their numbers increased twofold to fourfold with Triton- or Tween-treated virus and with nucleocapsids (see Fig. 3). Again, they seemed to be sensitive to mild virus challenge. Eccentric-nuclei cells were abundant in nonstimulated cultures (2000 cells/well), but their numbers were not increased with the addition of isolated viral proteins, and only a two-fold increase could be detected using Tween-treated virus. The adherent cells, although present in nonstimulated cultures (2000 cells/well) were increased about twofold to threefold by the addition of the G and N proteins, respectively (Fig. 4). Similar behavior was observed after addition of Tween-treated virus and isolated nucleocapsids (Fig. 3). The highest number of adherent cells (10,000/well) was obtained when the N-stimulated cultures were challenged with virus. Also, the cells in these cultures increased their average diameter to 64 ± 24 μm (n = 30).

DISCUSSION

In this report, we describe the stimulation of adherent cells from the kidney of rainbow trout by the addition of purified G or N (Nx) proteins of the rhabdovirus causing viral hemorrhagic septicemia in this species. The addition of M_1 or M_2 did not stimulate these same cultures.

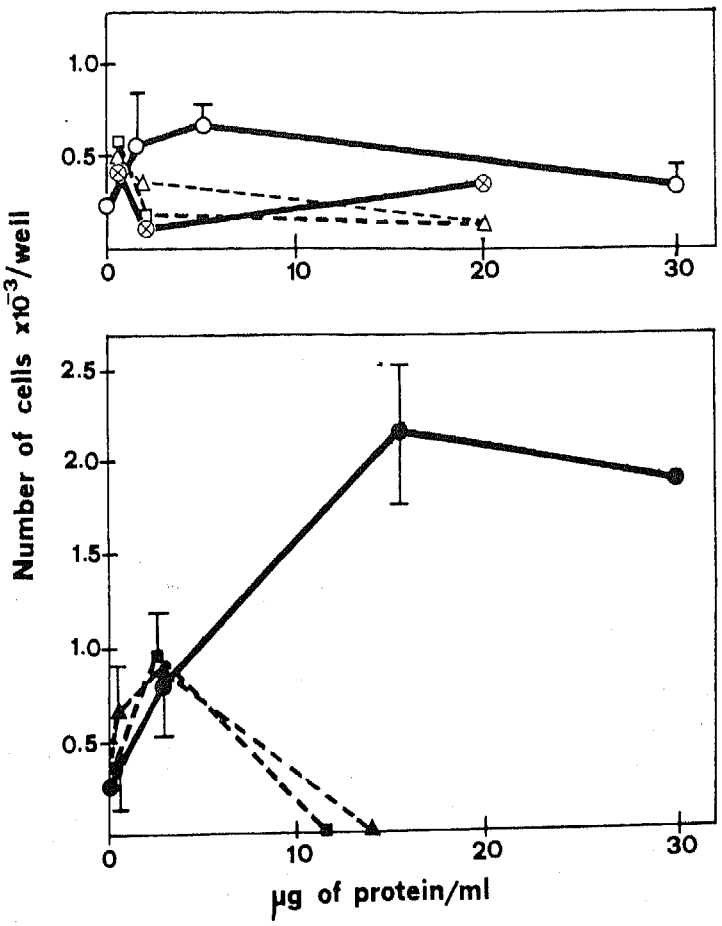

FIG. 2. Adherent cells stimulated by addition of isolated VHS viral proteins. Cells (24,000/well) were cultured in presence of proteins at 20°C for 11 days, then at 14°C for 11 days, and harvested. Averages and standard deviations from four fields counted in four different wells are represented. ●, G; ■, N; ▲, Nx; □, M_1; △, M_2; ○, heat-killed VHS virus; ⊗, heat-killed IPN virus.

Attention to the induction of protective antiviral immunity has focused primarily on the response to the surface glycoprotein G because of the observation that neutralizing antibody to VHS virus shows exclusive specificity for the G protein (3,12,14). Therefore, the stimulation of leukocytes by the addition of G protein was not unexpected; however, both N and Nx also had a stimulatory effect that was even greater than that of G in VHS-immunized trout, suggesting that the N protein stimulated an immunologic memory rather than a primary reaction *in vitro*, as seems to happen with the rabies (10) and vesicular stomatitis (16) viruses.

In contrast to the rhabdovirus G protein, which shows a higher degree of antigenic variability, the N protein of salmonid rhabdovirus is more conserved, as was shown by using a pannel of Mabs (manuscript in preparation). On the other hand, the VHS virus ribonucleoprotein might be able to induce a protective immune response against lethal challenge with VHS virus, as it happens in rabies (10). In addition, the VHS virus ribonucleoprotein could be a major target antigen for T-helper cells to assist a neutralizing antibody response of trout immune B-like lymphocytes. Whether this type of interaction might be occurring in the mixed cultures described here to allow the adherent cells to respond to challenge with live virus is not known at present. Because resting lymphocytes become activated only when they find their antigen in the presence of macrophage-like cells, the results obtained could indicate that adherent cells (macrophage-like cells) are

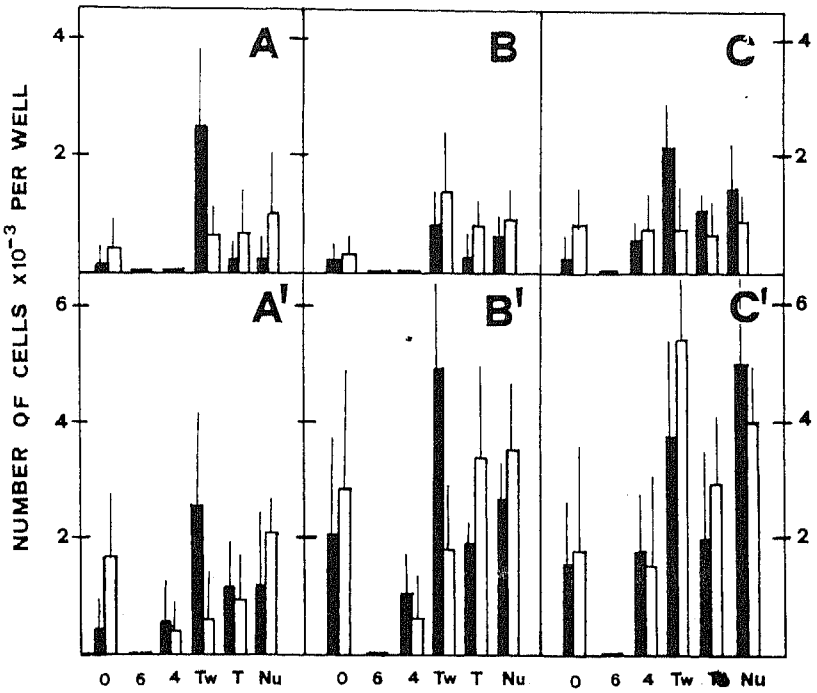

FIG. 3. Trout kidney cells cultured with tween-treated VHS virus. Cells (24,000/well in 100 μl) were cultured at 20°C for 9 days, challenged, and cultured at 14°C for 7 days. They were cultured in the presence of 10^6 or 10^4 PFU per ml of VHS (6 or 4, respectively), 65 μg/ml of Tween-treated VHS, 2 μg/ml of Triton-treated VHS, and 1 μg/ml of VHS nucleocapsids or in their absence (0). A, B, C: Cells from trout injected with Freund's. A′, B′, C′: Cells from trout injected with purified VHS virus in Freund's. A, A′: Multinucleated cells; B, B′: Eccentric-nuclei cells; C, C′: Adherent cells. Black bars, = number of cells after 14 days of culture in presence of treated virus. White bars=number of cells after same cultures were challenged on the 9th day with 10^6 PFU of VHS virus per ml. Averages (bars) and standard deviations (vertical lines) from four fields counted in four different wells are represented.

nonspecifically activated by the viral proteins, especially when the cells are from immunized trout (increase in number and spreading) to be able later to help trout lymphocytes in a specific response (antibodies and/or cytotoxicity). They might resist challenge because they are a nontarget cell for the VHS virus, as it is probably that only dividing lymphocytes are the target of this virus (9), or because we employed mild conditions of infection. For instance, when we used a higher pH (discharging the CO_2 after 3–4 days of infection), only a few surviving cells could be found in the cultures (data not shown).

More unexpected was the fact that the addition of the G and, to a lesser extent, the N proteins was stimulatory, not only for leukocytes from immunized trout (7,8) but also for leukocytes from nonimmunized trout. Because some of the targets of VHS virus seem to be leukocytes (9), the possibility exists that the external G protein acts as a mitogen for the cells that the virus is then going to infect. On the other hand, the G and N proteins could be acting as adjuvants by increasing the number of virus-carrier cells or as a chemotaxis signal to attract and activate adherent cells to increase the dissemination of the infection throughout the body.

The development of *in vitro* immune response assays to study viral antigens or immunomodulators in teleosts is a strategy for preliminary screening of candidate immune response modifiers (genetically engineered viral proteins, for instance) before doing extensive *in vivo* experiments. However, which of the immunologic functions should be optimized? According to the results described here, we might be looking simply for the induction of the greatest increase in adherent cells in the leukocyte cultures. The morphology of the stimulated cells is similar to that of the adherent cells described previously by us (manuscript in

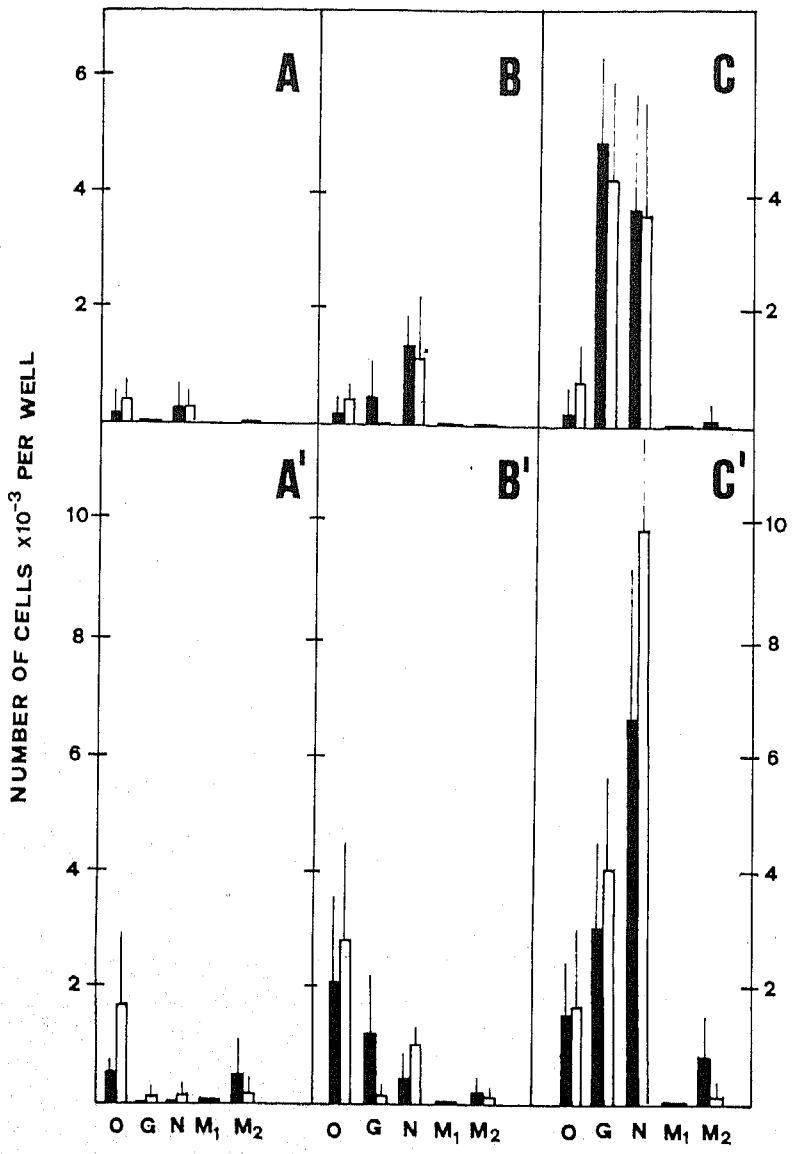

FIG. 4. Trout kidney cells cultured with purified VHS viral proteins. Cells (24,000/well in 100 μl) were cultured at 20°C for 9 days, challenged, and cultured at 14°C for 7 days in presence of 3 μg/ml of G, 1.5 μg/ml of N, 2.2 μg/ml of M_1, or 1 μg/ml of M_2 electroeluted proteins or in their absence (0). A, B, C: Cells from trout injected with Freund's. A′, B′, C′: Cells from trout injected with purified VHS virus in Freund's. A, A′: Multinucleated cells; B, B′: Eccentric-nuclei cells; C, C′: Adherent cells. Black bars=number of cells after 14 days of culture in the presence of electroeluted viral proteins. White bars=number of cells after the same cultures were challenged on 9th day with 10^6 PFU of VHS virus per ml. Averages (bars) and standard deviations (vertical lines) from four fields counted in four different wells are represented.

preparation) and to the macrophages or melanomacrophages described by Braun-Nesje and associates (5). The importance of macrophages (monocytes) as accessory cells in higher vertebrate immune responses is well established, but it is not clear at all what their function is in fish. Possibly, their increased presence is attributable to earlier viral protein-induced stimulation events, the adherent cells being the final effectors of the defense mechanism. The primitive model studied here (trout), however, might have both functions in only one cell (the adherent cell). Further studies are in progress to examine the earliest events after the addition of the G or the N proteins to the leukocyte cultures.

ACKNOWLEDGMENTS

Thanks are due to Dr. J. Jimenez, M. Babin, and Carmen Hernandez for their help with the Mabs used. Thanks are due also to Pilar Parrilla for help with the virus purification and to Dolores Frías for taking care of the aquarium. We appreciate the help of J. Coll Perez in typing and drawing.

This work was supported by Research Grant 8568 from the Instituto Nacional de Investigaciones Agrarias, Ministerio de Agricultura, Spain.

REFERENCES

1. Basurco, B., and J.M. Coll. 1989. Variabilidad del virus de la septicemia hemorragica viral de la trucha en España. Med. Vet. 6:425–430.
2. Basurco, B., and J.M. Coll. 1989. Spanish isolates and reference strains of viral haemorrhagic septicaemia virus show similar protein size patterns. Bull. Eur. Assoc. Fish Pathol. 9:92–95.
3. Bernard, J., P. De Kinkelin, and M. Bearzotti-Le Berre. 1983. Viral haemorrhagic septicaemia of rainbow trout: relation between the G polypeptide and antibody production on protection of the fish after infection with the F25 attenuated variant. Infect. Immun. 39:7–14.
4. Bolognesi, D.P. 1990. Fresh pathways to follow. Nature 344:818–819.
5. Braun-Nesje, R., G. Kaplan, and R. Seljelid. 1982. Rainbow trout macrophages in vitro: morphology and phagocytic activity. Dev. Comp. Immunol. 6:281–291.
6. Bunschoten, H., R.J. Klapmuts, I. Claassen, S.D. Reyneveld, A.D.M.E. Osterhaus, and F.G.C.M. Uytedehaag. 1989. Rabies virus-specific human T cell clones provide help for an in vitro antibody response against neutralizing antibody-inducing determinants of the viral glycoprotein. J. Gen. Virol. 70:1513–1521.
7. Chilmonczyk, S. 1977. Stimulations specifiques des lymphocytes de truites arc-en-ciel résistantes à la SHV. Bull. Off. Int. Epiz. 87:395–396.
8. Chilmonczyk, S. 1978. Stimulation specifique des lymphocytes de truites arc-en-ciel (Salmo gairdneri) résistantes à la septicemie hemorrhagique viral. C. R. Acad. Sci. Paris 287:387–389.
9. Chilmonczyk, S., and E. Oui. 1988. The effects of gamma irradiation on the lymphoid organs of rainbow trout and subsequent susceptibility to fish pathogens. Vet. Immunol. Immunopathol. 18:173–180.
10. Dietzschold, B., H. Wang, C.E. Rupprecht, E. Celis, M. Tollis, H. Ertl, E.H. Katz, and H. Koprowski. 1987. Induction of protective immunity against rabies by immunization with rabies virus ribonucleoprotein. Proc. Natl. Acad. Sci. U.S.A. 84:9165–9169.
11. Ertl, H.C.J., B. Dietzschold, M. Gore, L. Otvos, J.K. Larson, W.H. Wunner, and H. Koprowski. 1989. Induction of rabies virus-specific T-helper cells by synthetic peptides that carry dominant T-helper cell epitopes of the viral ribonucleoprotein. J. Virol. 63:2885–2892.
12. Gilmore, R.D., Jr., H.M. Engelking, D.S. Manning, and J.C. Leong. 1988. Expression in Escherichia coli of an epitope of the glycoprotein of infectious hematopoietic necrosis virus protects against challenge. BioTechnology 6:295–300.
13. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.
14. Lorenzen, N., N. J. Olesen, and P.E. Vestergard-Jorgensen. 1990. Neutralization of Egtved virus pathogenicity to cell cultures and fish by monoclonal antibodies to the viral G protein. J. Gen. Virol. 71:561–567.
15. Martinez, J., and J.M. Coll. 1988. Selection and clinical performance of monoclonal anti-C-reactive protein in ELISA quantitative assay. Clin. Chim. Acta 176:123–132.
16. Puddington, L., M.J. Bevan, J.K. Rose, and L. Lefrancois. 1986. N protein is the predominant antigen recognized by vesicular stomatitis virus specific cytotoxic T cells. J. Virol. 60:708–717.
17. Rueda, A., and J.M. Coll. 1988. Cloning of myelomas and hybridomas in fibrin-clots. J. Immunol. Meth. 114:213–217.

18. Sanchez, M.C.T., and J.M. Coll. 1989. La estructura de las immunoglobulinas de peces. Immunologika 8:47–54.

19. Sanchez, C., Dominguez, J., and J.M. Coll. 1989. Immunoglobulin heterogeneity in the trout Salmo gairdneri (Richardson). J. Fish Dis. 12:459–465.

Address reprint requests to:
J.M. Coll
Departamento de Sanidad Animal
INIA-CRIDA 06
Embajadores, 68
28012 Madrid, Spain