STUDIES OF TGEV SPIKE PROTEIN GP195 EXPRESSED IN *E. COLI* AND BY A TGE-VACCINIA VIRUS RECOMBINANT

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ABSTRACT

The gene coding for the surface spike protein gp195 of TGEV has been cloned and expressed in *E. coli* in the form of fusion proteins. These proteins were isolated and used to immunize laboratory animals. All animals developed antibodies cross-reacting with the TGEV virion but failed to neutralize the virus. The entire gp195 gene was also inserted into vaccinia to generate a TGEV-vaccinia recombinant virus (vTGE) that expressed TGEV gp195. Animals vaccinated with vTGE produced neutralizing antibodies against both TGEV and vaccinia. These results suggest the potential use of the recombinant vTGE as a vaccine against TGEV infection.

INTRODUCTION

Transmissible gastroenteritis (TGE) is a worldwide enteric disease of swine. Pigs of all ages can be affected, but the disease is especially serious in newborn pigs, with a very high mortality rate. Current vaccines rely on the transmission of lactogenic immunity from vaccinated sows to nursing piglets and have not been very effective in practice (Bohl, 1981).

The disease is caused by a virus (TGEV) that belongs to the coronavirus family. Like other coronaviruses, the TGE virus particle contains three major structural proteins. The nucleocapsid protein, p50, (molecular weight 50,000 daltons) is associated with the genomic RNA, the matrix protein, gp31, is a glycoprotein that is mostly embedded in the viral membrane (Rottier, et al., 1984), and the spike protein, gp195, forms the characteristic knob-like structures which protrude from the surface of the viral envelope.

M. Z. Atassi et al. (eds.), *Immunobiology of Proteins and Peptides—III*
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It has been shown that tunicamycin treatment of cells infected with murine or avian coronaviruses results in the production of non-infectious virions that lack the spike protein. (Holmes, et al., 1981; Niemann and Klenk, 1981; Rottier, et al., 1981; Stern and Sefton, 1982). Garwes et al. (1978/1979) reported that a TGEV subviral component consisting mainly of gp195 could elicit a neutralizing immune response when injected into pigs. These observations suggested that gp195 was important both for viral infectivity and for neutralization. We have reported (Hu, et al., 1984) the identification of six discrete TGE viral mRNAs with sizes ranging from 2 to 19 kb (kilobases). The gene coding for gp195 was mapped on the 5' half of the 9 kb mRNA. The entire gene of gp195 was cloned and expressed at high levels in E. coli. In this work, we describe the isolation of these proteins from E. coli and their antigenic properties, particularly whether they are capable of inducing a neutralizing response in animals.

Recently, the use of vaccinia virus as a vector for expressing foreign genes has led to the concept of a novel type of live vaccine. (Mackett et al., 1984). Recombinant vaccinia viruses containing the herpes simplex virus surface antigen (Paoletti et al., 1984) or the influenza hemagglutinin gene (Smith et al., 1984) have been shown to render the vaccinated animals resistant to the corresponding infectious virus challenge. The broad host range of vaccinia enables the application of such live vaccines in domestic animals such as pigs.

To test this possibility, we constructed a TGE-vaccinia hybrid virus by inserting the TGEV gp195 gene into vaccinia virus. The product of gp195 was detected in animal cells infected with the hybrid viruses by immunoprecipitation and by immunoperoxidase staining with TGEV specific antiserum. Mice inoculated with this TGE-vaccinia recombinant virus elicited neutralizing antibodies against both TGEV and vaccinia virus.

MATERIALS AND METHODS

Viruses and Cells

The virulent Miller strain TGEV (American Tissue Culture Collection VR-743) was propagated in swine testes (ST) cells as described by McClurkin (1965). Vaccinia virus used in this study was the WR strain (ATCC VR-119). African green monkey cells (CV-1) were used for the growth of vaccinia virus. Human TK^- (line 143) cells were used for selecting TK^- vaccinia recombinant virus.

Endoglycosidase H digestion

Endoglycosidase H (37IU/mg) was obtained from Miles. 50 µg of purified TGEV was digested with 5 mU of enzyme in 100 µl of 150 mM sodium citrate buffer (pH 5.5) at 37°C for 16 hrs. An aliquot of the product was tested on SDS-polyacrylamide gel for complete digestion.

Animal immunization

Rabbits, rats and mice have been used for testing the antigenicity of *E. coli* expressed TGEV proteins. Animals were given five subcutaneous immunizations at two week intervals. The first immunization was with complete, and subsequent four boosters were with incomplete Freund's adjuvant. Antiserum was collected after the third and last injection.

For the TGE-vaccinia recombinant virus studies, female Balb/C mice were inoculated by dabbing purified virus (at 10^9 pfu/ml) onto wounds made by scratching the base of the tail. Mice were bled at weeks 2, 4, 6 and 8 post-inoculation of vaccine and their sera were tested for antibodies against vaccinia and TGEV.

Antibody neutralization

Pre-immune and immune sera were heat-inactivated, mixed with an equal volume of virus, incubated at 37°C for 1 hour, and plated on cells. After one hour of adsorption, the monolayer was then washed twice with phosphate buffered saline (PBS) and overlaid with agar. Plaques were scored by staining with neutral red after 48 hr.

[^{35}]-methionine labeling of cells and immunoprecipitation.

About 8 to 10 hr after infection, cells were pre-incubated in methionine deficient medium for 30 min., then labeled with 50 µCi/ml [^{35}S]-methionine for 1 hr. The monolayer was washed with PBS and lysed with ice-cold disruption buffer containing 0.5% Nonidet P-40, 1% deoxy-cholate, 50mM NaCl, and 25 mM Tris (pH 8.0). Cell debris was removed by centrifugation at 6,000 g for 15 min. For immunoprecipitation, 250 to 500 µl of labeled cell lysate was mixed with 5 to 10 µl of antiserum and incubated at 37°C for 1 hr followed by overnight incubation at 4°C. 100 µl of formalin-treated staphylococcal cells (Pansorbin, Calbiochem-Behring) was added and the incubation was continued at 4°C for 45 min. The immunoprecipitate was washed three times in the solutions described by Oppermann et al. (1979) and analyzed by electrophoresis through a 6 to 18% gradient polyacrylamide gel using the buffer system described by Laemmli (1970). Fluorography of the gel was performed using EN^{3}hance (New England Nuclear) following manufacturer's procedure.

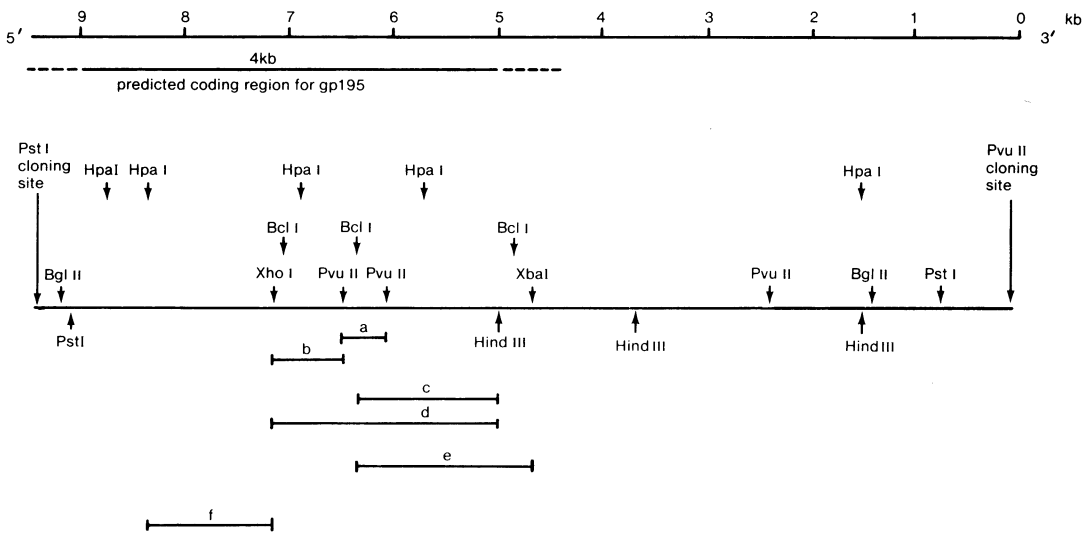

Figure 1. Restriction endonuclease cleavage map of a 9.4 kb TGEV cDNA clone. Nucleic acid sequence analysis has revealed one continuous, unique, translation open-reading frame coding for gp195, which starts at 8 bases downstream from the second 5' HpaI site and ends at 80 bases upstream from the XbaI site. Fragments a through f have been subcloned for expression in E. coli.

Immunoperoxidase staining

Infected cells were fixed with 2% para-formaldehyde followed by cold acetic acid-ethanol (5/95%) and processed for immunoperoxidase staining using (i) a porcine hyperimmune serum to TGEV (M-20, from Dr. L. Saif, Ohio State University) diluted 1:200, and (ii) a rabbit HRP (horse radish peroxidase) conjugate against porcine immunoglobulin, diluted 1:200 (Dako). When the test serum is of mouse origin, a goat HRP conjugate against mouse IgG (Boehringer-Mannheim) was used.

RESULTS

E. coli expression of TGEV gene gp195

We have previously (Hu et al., 1984) reported the cloning and expression of the TGEV gp195 gene in E. coli. Briefly, each restriction enzyme cleavage fragment (depicted as a-->f in Figure 1) of gene gp195 was ligated to another fragment with the same translation reading frame which codes for the amino terminus of a second protein. This resulted in the formation of fusion proteins. Such fusion proteins were produced under the regulation of the copy number runaway plasmid pCFM414 in E. coli at high levels. In all cases, these proteins were sequestered in an insoluble matrix that became visible under light microscope at 2 to 3 hours after induction by temperature shift. Upon breakage of the E. coli cells with a French press apparatus, these proteins can be separated from most of the soluble cellular proteins by a short, low speed centrifugation. The pellet was harvested and subjected to various purification procedures described below.

Isolation of TGEV fusion proteins from E. coli

The inclusion bodies with which these TGEV fusion proteins were associated contain many other less abundant proteins, lipids and nucleic acids. A number of dissociation conditions were tested to release the proteins of interest from the inclusion bodies. We noticed that different fusion proteins could have quite variable biochemical properties thus requiring different isolation schemes.

Methods that have been used include:

(i) Alkali solubilization: The optimal pH required to solubilize each protein from the inclusion bodies without irreversibly denaturing it was determined by analyzing the insoluble pellet and soluble supernatant fractions after each alkali treatment. Usually at ~pH 12, these fusion proteins can be solubilized whereas the other abundant bacterial membrane proteins remain in the pellet. An example is shown in Figure 2.

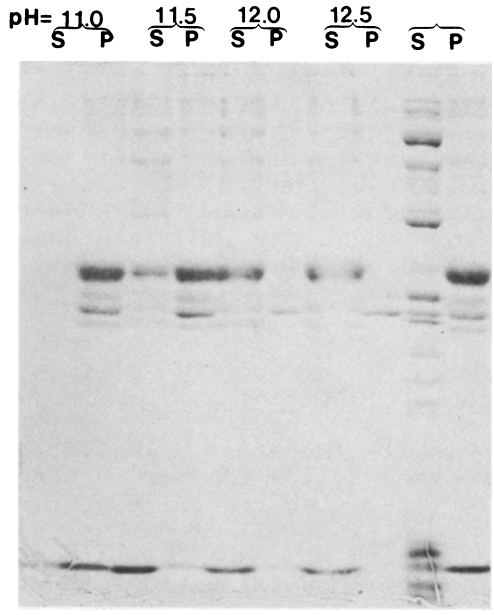

Figure 2. SDS-polyacrylamide gel depicting the solubilization process of E. coli expressed, TGE gp195 (fragment b) by alkali pH. The two lanes to the far right represent the protein content in the pellet (P) and the supernatant (S) fractions after a low speed centrifugation of the total E. coli cell extract. This pellet fraction was then adjusted to alkali pH as indicated and centrifuged to separate the soluble (S) from the insoluble (P) components.

(ii) Acid solubilization: We found that some of the proteins could tolerate and be solubilized in 50% acetic acid. After removing the insoluble material by centrifugation, the supernatant was applied on a gel filtration column such as Sephadex, and the proteins were eluted with 10% acetic acid. The fractions containing the proteins of interest were pooled and recovered by dialysis extensively against buffer.

(iii) A denaturing reagent such as urea could be used in conjunction with a reducing agent for solubilizing the proteins. Further purification could be achieved by the use of ion-exchange columns such as DEAE-and/or CM-Trisacryl (Reactifs IBF). Urea was then removed by dialysis against buffer.

(iv) Some of the proteins could be preferentially solubilized from the inclusion bodies with 1% Sarkosyl under non-reducing conditions (Figure 3). Solubilized

samples were fractionated through a gel filtration column using 1% Sarkosyl as eluent. High molecular weight aggregates came through in the excluded volume. Sarkosyl associated with the proteins from pooled fractions was removed by passing through an ion-retarding column, such as AG11-A8 (Bio-Rad).

We have frequently experienced aggregation problems while isolating these proteins. This is not unexpected because these proteins contain many hydrophobic regions, as predicted from the nucleic acid sequences, which is typical for membrane associated proteins. Furthermore, these proteins, when expressed in E. coli, lack the carbohydrate moieties that are fairly abundant in the native viral protein. Our previous experiments involving tunicamycin inhibition or endoglycosidase H digestion of gp195 (Hu et al., 1984) showed that about 50,000 daltons of this

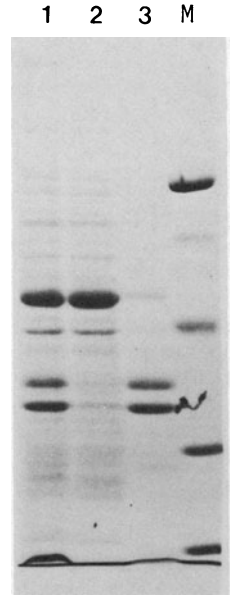

Figure 3. SDS-polyacrylamide gel pattern of (1) total proteins of inclusion bodies from E. coli expressing TGEV gp195 (fragment f). (2) Soluble and (3) insoluble components of (1) after the treatment with 1% sarkosyl in the absence of reducing reagent.

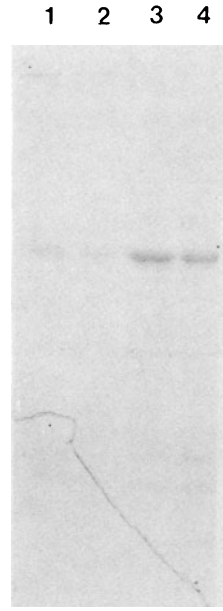

Figure 4. Nonreducing SDS-polyacrylamide gel of TGEV gp195 (fragment e) when the sample contains protein aggregates (lane 1,2) or monomers (lane 3,4).

protein was composed of carbohydrates. These carbohydrates could be necessary for maintaining gp195 or its fragments in solution. To determine the extent of aggregation occurring during purification of these proteins, we constantly compared their electrophoresis patterns on reducing gels versus non-reducing gels. The appearance of high molecular weight bands migrating as multimers, or material which failed to enter the non-reducing gel, was taken as indications of aggregates. One example of such comparison is depicted in Figure 4. The procedure that resulted in the lowest level of aggregates was chosen to isolate each protein.

(v) Preparative SDS-polyacrylamide gel electrophoresis: This procedure was only used to prepare a small amount of pure protein. Coomassie blue stained bands containing the proteins of interest were excised from the gel and subjected to electroelution. The recovered proteins were then dialyzed extensively to reduce the amount of associated SDS.

Neutralizing response induced by deglycosylated TGEV

We have previously demonstrated (Hu et al., 1984) that endoglycosidase H treatment converts gp195 and gp31 into proteins of apparent molecular weight 145,000 and 29,000 daltons respectively. In order to determine whether the neutralization site of gp195 resides in the carbohydrate side chains or in the polypeptides, we immunized mice with TGEV that had been digested to completion with endoglysodidase H. All immunized animals developed neutralizing antibodies against TGEV. This result is consistent with the hypothesis that the polypeptide structure represents the neutralization target. The carbohydrates could still be important for the proper folding of gp195 when the protein is initially synthesized. However, our data indicate that once the gp195 is assembled into the virion, removal of the carbohydrate does not disturb the neutralization site(s) of the molecule.

Antigenicity of E. coli expressed gp 195

To analyze the antigenic properties of the TGEV gp195 gene product expressed in various systems, we used two antisera directed specifically against TGEV. The polyvalent neutralizing serum, M-20, was from a hyperimmunized pig recovered from TGEV infection. In addition, we have isolated a mouse hybridoma clone, 5H2-7, from which monoclonal antibodies with neutralizing reactivity against TGEV were obtained. M-20 serum reacts with all of the three major structural proteins of TGEV, namely, p50, gp31 and gp195, whereas the monoclonal antibodies only react with gp195. In a standard ELISA test, all of the gp195 fusion proteins synthesized in *E. coli* could be recognized by M-20 antiserum, typical level of reactions are shown in Figure 5a. Two of the products (coded by fragment *b* and *d*) also reacted well with the monoclonal antiserums (Figure 5b). These two fragments have in common the region between the XhoI site and the nearest PvuII site. We believe that this region contains the neutralizing epitope recognized by this monoclonal antibody.

Analysis of immune sera

Sera from animals immunized with various *E. coli* expressed gp195 fusion proteins were collected. All of them contain antibodies that react with the immunogen as well as with native TGEV virion in an ELISA test (Figures 6a and 6b). Furthermore, these sera were all capable of immunoprecipitating specifically gp195 from [^{35}S]-methionine labeled, TGEV infected cell lysates, an example is shown in Figure 7. However, none of the immune sera had

significant levels of neutralizating antibody. We suspected that the attachment of carbohydrates is crucial for folding gp195 into its native structure and that without these carbohydrates, these E. coli expressed fusion proteins were not in the correct conformation around the neutralization sites to induce the appropriate immune responses.

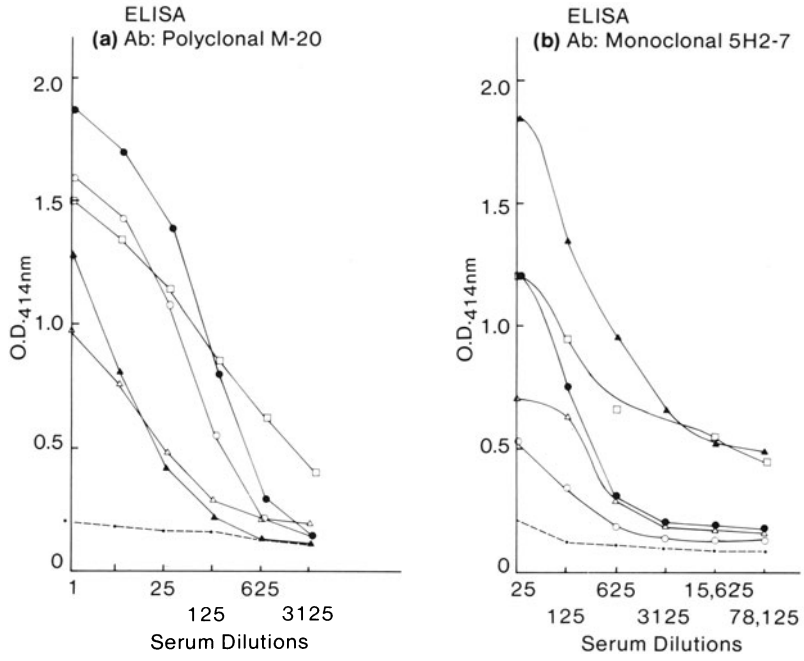

Figure 5. Binding characteristics of different E. coli expressed TGEV gp195 fragment to a polyvalent antiserum M-20 (a) or a monoclonal antiserum 5H2-7(b). Serial five-fold dilutions of antibody were incubated in the wells of microtiter plates containing adsorbed proteins from TGEV virion (▫), gp195 fragment b (▲), d (●), e (o), f (△), or control (-----) E. coli lysates. Bound antibodies were detected with HRP-labeled antiserum as described in the text.

Expression of gp195 in TGE-vaccinia recombinant virus (vTGE)

To construct a TGE-vaccinia recombinant virus, the DNA fragment carrying the entire TGEV gp195 gene (starting at the second 5' HpaI site and ending at the unique XbaI site as shown in Figure 1) and including the putative signal sequences was inserted into the BamHI cloning site of a

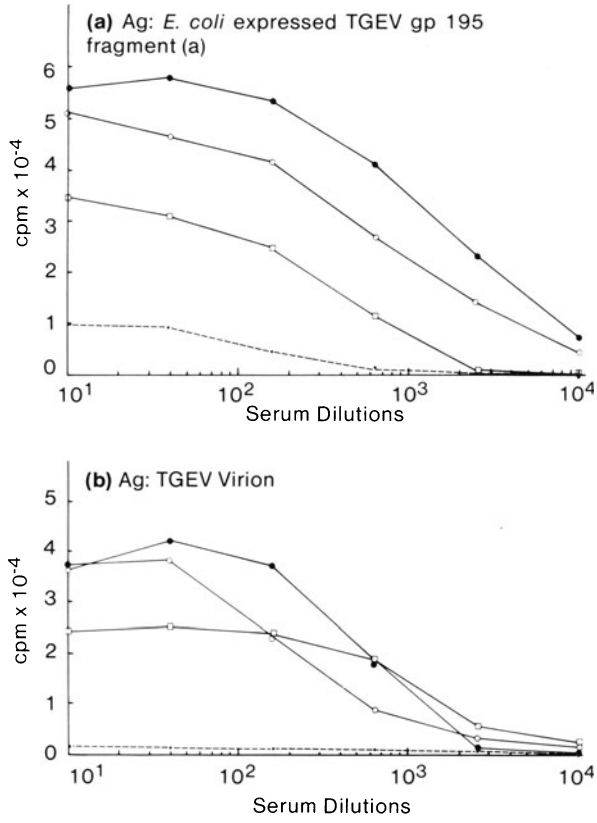

Figure 6. Specific binding of antibodies from two rabbits immunized with E. coli expressed TGEV gp195 fragment a (●, o). The microtiter plates were preabsorbed with either the immunogen (a) or intact TGEV virion (b). The polyvalent antiserum M-20 was used as positive control (□) and the preimmune rabbit serum was the negative control (-----). Bound antibodies were quantitized with ^{125}I-labeled staphalococcal protein A.

vaccinia expression vector, pGS20 (Mackett et al., 1984) after BamHI linker was added to the insert DNA. The insert was oriented to place its first ATG immediately downstream from the vaccinia 7.5 K gene promoter. This chimeric gene is in turn flanked by the vaccinia TK (thymidine kinase) gene (Figure 8). Recombinant viruses were selected on the basis of their TK^- phenotype after transfecting the constructed plasmid DNA into TK^- cells previously infected with the wild type vaccinia virus.

Cultures individually infected with viruses picked from separate TK^- plaques were further examined for the expression of gp195 by immunoperoxidase staining using

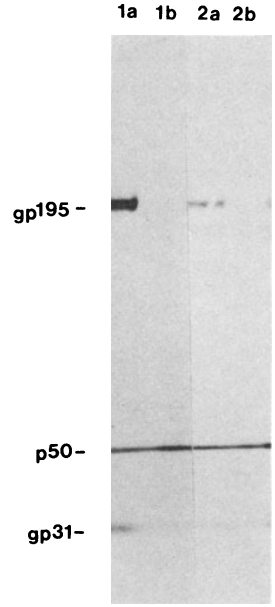

Figure 7. SDS-polyacrylamide gel analysis of TGEV proteins immunoprecipitated from infected cells with neutralizing pig serum M-20 (1a) and normal pig serum (1b), or with serum from rabbit immunized with E. coli expressed TGEV gp195 (2a) and preimmune rabbit serum (2b). Notice that M-20 recognizes all of the three major TGEV proteins whereas the rabbit immune serum only recognizes gp195.

TGEV-specific antiserum. Positive staining appeared at 12 hours after infection in the cytoplasm of 1/12 of the cultures indicating the infection by TGE-vaccinia recombinant virus (vTGE). Vaccinia viruses which obtained the TK$^-$ phenotype due to spontaneous mutation in the viral genome do not lead to positive staining of infected cells and can be easily recognized and discarded. The genuine vTGE virus was then subjected to several rounds of plaque purification. The stability of the recombinant virus and its ability to express TGEV gp195 was not altered after multiple passages in culture. The infectivity of vTGE is comparable to that of the wild type vaccinia virus in a number of cell lines that have been tested.

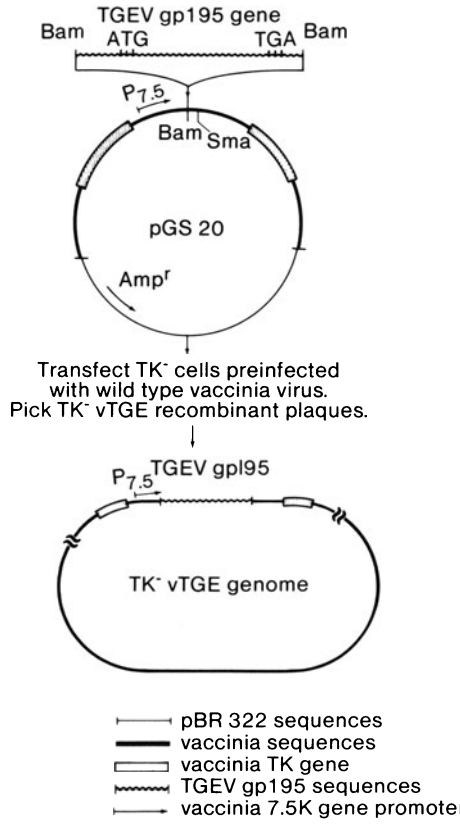

Figure 8. Construction of a TGE-vaccinia recombinant virus. The complete TGEV gp195 gene is inserted into vaccinia virus under the control of the 7.5K gene promoter. Recombinant viruses were generated as the result of homologous recombination between the transfer vector and the genomic DNA of vaccinia and was selected on the basis of their TK$^-$ phenotype.

The product of the inserted gp195 gene in vTGE-infected cells was analyzed by immunoprecipitation. As shown in Figure 9, a unique protein of apparent molecular weight 185K daltons was specifically precipitated by TGEV antiserum. The discrepancy in molecular weight with the native gp195 could reflect the difference in glycosylation between the two virus systems.

The 185K dalton protein represents a minor component when compared with the vaccinia virus-derived proteins. This is not surprising since the 7.5K gene promoter is not a strong promoter. Nevertheless, the amount of the 185K dalton protein is still detectable even in the whole cell lysate as shown in Figure 9.

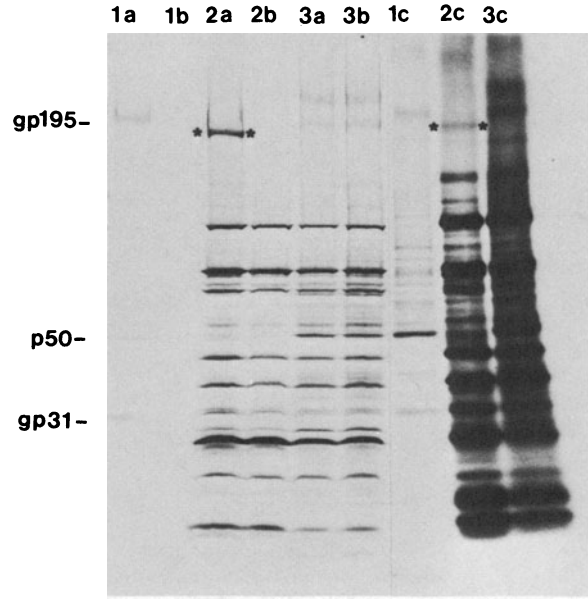

Figure 9. SDS-polyacrylamide gel analysis of immune-precipitated proteins from (1) ST cells infected with TGEV (2) CV-1 cells infected with recombinant vTGE and (3) CV-1 cells infected with vaccinia virus. Lane a's were precipitated with antiserum M-20, lane b's were precipitated with normal control serum, lane c's were total cytosol extract from corresponding infected cells.

TABLE 1

Mice # (Jackson Balb/C)	# Weeks Post inoculation* / Neutralizing titer**	2	4	6	2	4	6
		@ vaccinia			@ TGEV		
1898	vaccinia	40	100	400	<20	<20	<20
1899	vaccinia	60	100	400	<20	<20	<40
1989	vaccinia	70	150	380	<20	<20	<20
1990	TGE/vaccinia recombinant	60	130	345	<20	40	110
1991	TGE/vaccinia recombinant	70	70	75	<20	60	50
1992	TGE/vaccinia recombinant	50	60	350	<20	65	120
1993	TGE/vaccinia recombinant	40	70	370	<20	50	60
1994	TGE/vaccinia recombinant	65	60	250	<20	50	60
1995	TGE/vaccinia recombinat	55	175	400	<20	50	160
1996	TGE/vaccinia recombinant	60	135	350	<20	50	120

* Inoculation was done by dabbing a tail scratch with purified viruses with titer of 10^9 pfu/ml.

** Neutralizing titer was determined by plaque reduction assay, shown as the reciprocal of serum dilution that reduces 50% of the plaques.

Preimmune serum from each mouse was included in each assay as control.

We also used the technique of immunoperoxidase staining to analyze the distribution of gp195 in the cells. Both the polyvalent M-20 and the monoclonal 5H2-7 sera stained positively fixed cells that have been infected with TGEV or the recombinant vTGE. However, gp195 was found only on the surface of TGEV infected cells but not on vTGE infected cells, suggesting a possible difference in the transport pathway of gp195 in these two systems. We are currently investigating this hypothesis.

Immune response of mice vaccinated with vTGE

To determine whether vTGE could elicit a neutralizing antibody response in animals, mice were inoculated with vTGE or wild type vaccinia viruses by scarification. All mice developed local skin lesions typical of vaccinia infection, which healed quickly with no other clinical symptoms.

Figure 10 shows the relative binding titer of the representative mouse sera to TGEV and vaccinia virus at six weeks after inoculation using a standard ELISA test.

The neutralizing titer of these sera was determined by plaque reduction assay. As summarized in Table 1, neutralizing titer against vaccinia virus became detectable in all animals at the second week and continued to increase for at least six weeks. Mice inoculated with vTGE all showed concurrent increasing neutralizing titer against TGEV. The neutralizing titer against TGEV has been low, in the range of 50 to 150 but is consistently higher than the control group. Subsequent booster scratches did not raise the titer significantly.

DISCUSSION

To develop a subunit vaccine for any viral disease, it is crucial to first identify the viral antigen(s) that is essential for viral infectivity, particularly in the step of adsorption to the receptors of the target cells. Furthermore, such antigen itself must be able to induce neutralizing antibody production in animals and to confer protection against infection by the virus.

Subviral component of TGEV derived from purified virus that demonstrated these properties has been reported (Garwes et al. 1978/1979; Gough et al., 1983) but the immunogens in these studies have not been well characterized and the quantity required for successful protection was high.

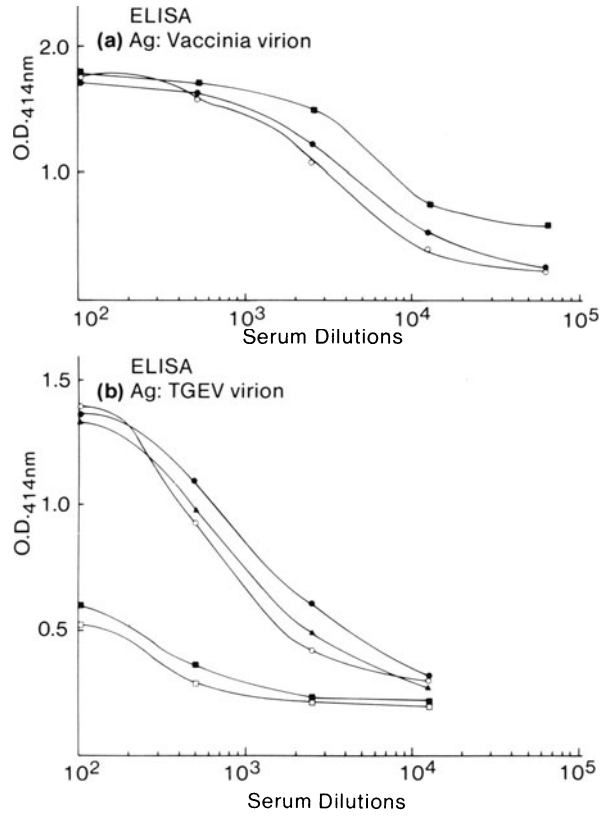

Figure 10. Antibody titer of mice inoculated with TGE-vaccinia recombinant virus (vTGE) (● , o , ▲) or a wild type vaccinia virus (■ , □) at six weeks using a standard ELISA test. Only the mice inoculated with vTGE elicit binding activity to TGEV in their sera, whereas the binding titers to vaccinia are comparable among all the mice.

The spike protein gp195 seems to be the most likely candidate for such a subunit vaccine because of its most accessible location on the virion. Our success with the TGE-vaccinia recombinant virus (vTGE) confirmed that gp195 alone will give rise to a TGEV-neutralizing immune response in animals.

We have prepared hybridoma cell lines derived from mice inoculated with TGEV and screened them with an *in vitro* neutralization assay. We found that all of the hybridoma clones that were secreting neutralizing antibodies reacted specifically with gp195. Our observation seems parallel to the studies reported by

Collins et al. (1982) on mouse hepatitis virus, namely, neutralization was mediated only by monoclonal antibodies which precipitated the spike protein (GP-1). Clearly, the spike protein plays an important role in virus attachment to the target cells in both cases.

It is disappointing that although the E. coli produced TGEV-fusion proteins elicited antibodies that reacted with the immunogen and intact viruses, they do not neutralize the infectivity of the virus. It appears that the structure of the E. coli produced protein is different and results in altered, "inactivated" neutralization epitopes. One possible explanation is that the addition of carbohydrates is essential for membrane glycoproteins such as gp195 to maintain their conformation. We are currently expressing gp195 in yeast and mammalian systems in an attempt to obtain material in its native conformation for testing.

It has been noticed that natural infection of sows with virulent TGE virus stimulates production of effective secretory antibodies but intramuscular inoculation of the pathogen generally does not (Bohl et al, 1972; Bohl and Saif, 1975). Since suckling pigs are protected only as a result of the frequent ingestion of colostrum or milk that contains TGEV-neutralizing antibodies, antigenic stimulation of the intestinal tract of the sows is a prerequisite for conferring lactogenic immunity to the piglets.

Recently, Smith et al. (1983) reported that hamsters intradermally vaccinated with a recombinant vaccinia virus carrying the influenza hemagglutinin (HA) gene produced sufficient local immunity to render the animals resistant to intranasal challenge of infectious influenza virus. Such protection is also believed to be greatly dependent on the secretory IgA antibodies. Their data encourages an approach in the control of TGEV infections by the use of the TGE-vaccinia recombinant virus. We are currently carrying out protection studies in pigs to examine the efficacy of the use of vTGE as a vaccine.

ACKNOWLEDGEMENT

We thank Dr. Margery Nicolson for advising the hybridoma work, Dr. Bernard Moss for providing pGS20 plasmid and Dr. Linda Saif for M-20 pig serum. We also thank Ms. Joan Bennett for preparation of the manuscript.

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