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Homologous and heterologous glycoproteins induce protection against Junin virus challenge in guinea pigs

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Tacaribe virus (TACV) is an arenavirus that is genetically and antigenically closely related to Junin virus (JUNV), the aetiological agent of Argentine haemorrhagic fever (AHF). It is well established that TACV protects experimental animals fully against an otherwise lethal challenge with JUNV. To gain information on the nature of the antigens involved in cross-protection, recombinant vaccinia viruses were constructed that express the glycoprotein precursor (VV–GTac) or the nucleocapsid protein (VV–N) of TACV. TACV proteins expressed by vaccinia virus were indistinguishable from authentic virus proteins by gel electrophoresis. Guinea pigs inoculated with VV–GTac or VV–N elicited antibodies that immunoprecipitated authentic TACV proteins. Antibodies generated by VV–GTac neutralized TACV infectivity. Levels of antibodies after priming and boosting with recombinant vaccinia virus were comparable to those elicited in TACV infection. To evaluate the ability of recombinant vaccinia virus to protect against experimental AHF, guinea pigs were challenged with lethal doses of JUNV. Fifty per cent of the animals immunized with VV–GTac survived, whereas all animals inoculated with VV–N or vaccinia virus died. Having established that the heterologous glycoprotein protects against JUNV challenge, a recombinant vaccinia virus was constructed that expresses JUNV glycoprotein precursor (VV–GJun). The size and reactivity to monoclonal antibodies of the vaccinia virus-expressed and authentic JUNV glycoproteins were indistinguishable. Seventy-two per cent of the animals inoculated with two doses of VV–GJun survived lethal JUNV challenge. Protection with either VV–GJun or VV–GTac occurred in the presence of low or undetectable levels of neutralizing antibodies to JUNV.

Introduction

Arenaviruses are enveloped viruses with genomes consisting of two single-stranded RNA segments designated small (S) and large (L), each of which contains two genes in an ambisense orientation. The S RNA encodes the nucleocapsid protein (N) at its 3′ end in the genome-complementary sense and the glycoprotein precursor (GPC) of the structural proteins G1/G2 at the 5′ end in the genome sense. Similarly, the L RNA encodes the virus polymerase (L protein) at the 3′ end and the Z (p11) protein at the 5′ end (Auperin *et al.*, 1984; Salvato, 1993; Franze-Fernández *et al.*, 1993; Djavani *et al.*, 1997).

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Two groups of arenaviruses are currently recognized (Buchmeier *et al.*, 1995). The Old World group, which includes among others lymphocytic choriomeningitis virus (LCMV) and Lassa virus (LASV), and the New World (Tacaribe complex) group. The prototype of the New World group, Tacaribe virus (TACV), seems not to be pathogenic, but the group also includes several viruses associated with severe haemorrhagic disease in humans. Junin virus (JUNV) causes Argentine haemorrhagic fever (AHF) (Parodi *et al.*, 1958), which has been recognized as a major public health problem in certain agricultural areas of Argentina. Machupo virus (MACV), first recognized in 1965 (Johnson *et al.*, 1965), has caused periodic outbreaks of haemorrhagic fever in Bolivia. In the 1990s, Guanarito virus (GUAV) and Sabia virus (SABV) have emerged as the aetiological agents of severe haem-

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orrhagic fever in Venezuela and Brazil, respectively (Salas *et al.*, 1991; Coimbra *et al.*, 1994).

A phylogeny of New World arenaviruses constructed on the basis of N gene sequences indicated that these viruses formed three lineages. TACV and the highly pathogenic JUNV, MACV, GUAV and SABV are all members of one of the lineages, with JUNV, MACV and TACV included in a sublineage (Bowen *et al.*, 1996). The phylogeny correlates well with the close antigenic relationships and cross-protection described among JUNV, MACV and TACV (Peters *et al.*, 1987; Martinez Peralta *et al.*, 1993).

Most cross-protection studies of New World arenaviruses have been directed towards TACV and JUNV, using guinea pigs and marmosets as experimental animals. Guinea pigs infected with the prototype JUNV XJ strain develop a lethal disease that shares many clinical and pathological features with AHF. Inoculation with a single dose of TACV protects guinea pigs fully against lethal JUNV infection (Peters *et al.*, 1987; Weissenbacher *et al.*, 1987; Martinez Peralta *et al.*, 1993).

Although a considerable number of studies on cross-protection have been reported, there is so far no information on the nature of the antigens involved. However, this knowledge could have relevance to the design of a vaccine against multiple New World arenaviruses. In order to get an insight into this point, we constructed vaccinia virus recombinants that express the TACV GPC or N proteins and studied both the immunogenic properties and the ability of the recombinants to elicit a protective immune response against lethal challenge with JUNV in guinea pigs. We also constructed a vaccinia virus recombinant that expresses JUNV GPC, and the immunogenicity and protective efficacy of this recombinant were evaluated.

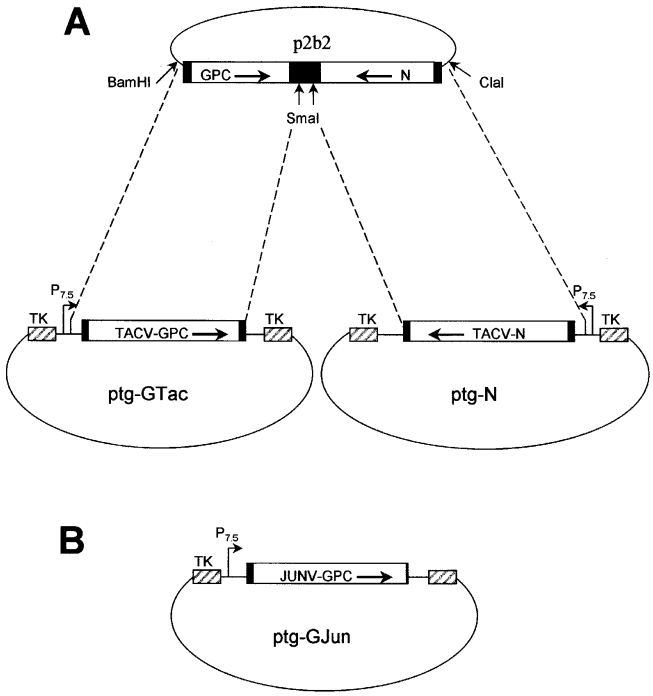

Fig. 1. Construction of transfer vectors for recombination with vaccinia virus. Transfer vectors were constructed as detailed in Methods. (A) Plasmid p2b2 (Franze-Fernández *et al.*, 1987) contains the entire TACV S RNA sequence except 17 nucleotides at the 5′ end. Arrows indicate the 5′ to 3′ orientation of the TACV GPC and N genes. Non-coding S RNA sequences are indicated in black. *Bam*HI and *Cla*I are PAT153/PvuII/8 sites. (B) Schematic representation of the transfer vector including JUNV GPC. The orientation of the gene and non-coding S RNA sequences are denoted as in (A). In the transfer vectors, TK denotes vaccinia virus thymidine kinase sequences and $P_{7.5}$ indicates the position of the vaccinia virus promoter.

Methods

■ **Construction of transfer vectors and recombination with Copenhagen vaccinia virus.** Chimeric transfer vectors containing TACV genes were constructed as indicated in Fig. 1(A). Both the TACV GPC and N genes were obtained from plasmid p2b2. This plasmid contains the entire TACV S RNA sequence minus 17 nucleotides at the 5′ end of the genomic S RNA (Franze-Fernández *et al.*, 1987). To obtain the transfer vector ptg-N, the cloned N gene was excised by restriction with *Cla*I followed by Klenow treatment to fill in the end and subsequent digestion with *Sma*I. The fragment was electrophoresed in an agarose gel and the band was purified from the gel and ligated blunt-ended into the multiple cloning site of the transfer vector ptg186-poly (Kieny *et al.*, 1984), previously cleaved with *Sma*I. The orientation of the gene in the chimeric vector was determined by restriction enzyme digestion. A transfer vector containing the TACV GPC gene (ptg-GTac) was constructed by cutting plasmid p2b2 with *Bam*HI and *Sma*I and ligation of the purified fragment into ptg186-poly vector digested with the same enzymes.

For construction of a transfer vector containing the JUNV glycoprotein, the JUNV GPC gene was first amplified by RT–PCR. To this end, Vero cell monolayers were infected with JUNV XJ strain (m.o.i. 0·1) for 96 h and, at this time, cells were removed by scraping and total RNA was purified by the method of Chomczynski & Sacchi (1987). Primers for RT–PCR were designed based on reported sequences. The forward primer was 5′ tagctgcagCGCACCGGGGATCCTAGG 3′. Residues in upper case correspond to the conserved 5′ terminus of the arenavirus S genome (Auperin *et al.*, 1984) and those in lower case were added to generate a *Pst*I site for cloning purposes. The reverse primer (5′ gctccCGGGAGGTCTTAGTGTCCTCTAC 3′) included a sequence (in upper case) that corresponded to positions 1520–1542 of the JUNV S genome (GenBank accession no. D10072) plus nucleotides (in lower case) designed to generate a *Sma*I site for cloning purposes. The RT–PCR amplification product was size-fractionated by gel electrophoresis and the band was purified from the gel and sequenced directly with the *fmol*-DNA sequencing system (Promega). Comparison of the sequence with that reported for JUNV XJ strain GPC (GenBank accession no. U70799) revealed 51 changes at the nucleotide level and eight amino acid changes, all within the G1 region. The changes were confirmed by sequencing an independent RT–PCR amplification product directly (data not shown). For construction of the transfer vector containing the JUNV GPC gene (ptg-GJun), the purified RT–PCR fragment was digested with *Pst*I and *Sma*I and ligated into the ptg186-poly vector digested with the same enzymes (Fig. 1B). The nucleotide sequence of the JUNV GPC gene in the chimeric vector clone selected for recombination with vaccinia virus was verified. It was identical to that of the RT–PCR amplification fragment.

Homologous recombination by co-transfection of temperature-sensitive (ts7) vaccinia virus-infected CV-1 cells with chimeric vector and wild-type Copenhagen vaccinia virus DNA was carried out according to protocols described by Kieny *et al.* (1984). Recombinant viruses carrying the foreign gene in the TK locus of vaccinia virus were selected by three rounds of plaque-purification on HuTK$^-$ 143B cells (ATCC) grown in selective medium (DMEM containing 100 μg/ml 5-bromodeoxyuridine; Mackett *et al.*, 1985). Homogeneity of virus progeny was monitored by a plaque–immunoperoxidase assay (Avrameas, 1972). All virus plaques reacted with hyperimmune serum to either TACV or JUNV. The TK$^-$ vaccinia virus recombinants that expressed the glycoprotein or the nucleoprotein of TACV were termed VV–GTac and VV–N, respectively. That expressing JUNV GPC was named VV–GJun. Control non-recombinant TK$^-$ vaccinia virus was obtained by plaque purification in selective medium of spontaneous TK$^-$ mutants generated in transfections with wild-type vaccinia virus DNA. This TK$^-$ mutant virus was termed VV.

■ **Radiolabelling, immunoprecipitation and protein gel electrophoresis.** Infected CV-1 cells were labelled for 1 or 4 h (200 μCi/ml) or for 16 h (50 μCi/ml) with Express(^{35}S) (New England Nuclear). Labelled TACV particles were obtained from the supernatant medium of TACV-infected cells labelled for 16 h. The medium was clarified by centrifugation at low speed and virus particles were pelleted through a sucrose cushion (5%, w/v, sucrose; 100 mM NaCl; 1 mM EDTA; 10 mm Tris–HCl, pH 7·4) by centrifugation (35 000 r.p.m., 3 h) in a Beckman SW 65 rotor. Labelled polypeptides from cell lysates, TACV particles or supernatant medium from JUNV-infected cells were immunoprecipitated as described previously (Iapalucci *et al.*, 1994) with the following modifications: incubation with the antisera was for 1 h at 4 °C and the immune complexes were precipitated by incubation for 1 h at 4 °C with Protein A–Sepharose CL-4B (Sigma). Hyperimmune serum to TACV or to JUNV was raised in rabbits and used at 1 : 100 dilution. Monospecific sera to recombinant TACV proteins GPC and N, obtained as described previously (Rossi *et al.*, 1996), were used at 1 : 50 dilution. Monoclonal antibodies to JUNV glycoproteins were GB03-BE08, GDO1-AG02, QC03-BF11 and ODO1-AA09; those to JUNV N were SA02-BG12 and ICOG-BE10 (Sanchez *et al.*, 1989). All were obtained from A. Sanchez (Centers for Disease Control, Atlanta, GA, USA) and were used at 1 : 200 dilution. Analysis of the proteins by SDS–PAGE was performed as described previously (López & Franze-Fernández, 1985). Gels were dried and exposed to BioMax films by using the TranScreen-LE intensifying screen (Kodak).

■ **Virus neutralization assay.** Neutralizing antibodies (NA) against JUNV, TACV and vaccinia viruses in sera were determined by a plaque-reduction assay on Vero cells as described in Candurra *et al.* (1989) except that, in vaccinia virus-neutralization assays, plaques were developed at day 2. Neutralization titres are expressed as the reciprocal of the serum dilution that resulted in a 50% reduction in the number of plaques with respect to control serum.

■ **Immunization and challenge of guinea pigs with JUNV.** Outbred male guinea pigs weighing 300–400 g were inoculated subcutaneously on days 0 and 68 with 1×10^8 p.f.u. of the vaccinia recombinants or VV. The single inoculation (day 0) with 1×10^5 p.f.u. TACV was performed intramuscularly. On day 87, animals were challenged with 1×10^3 p.f.u. JUNV XJ strain by intramuscular inoculation. Clinical signs, weight and mortality were recorded for 30 days after challenge. Blood samples were collected by cardiac puncture.

Results

Generation of recombinant vaccinia viruses and characterization of the expressed proteins

Foreign genes were inserted into the TK locus of the Copenhagen strain of vaccinia virus downstream of the vaccinia virus early-late $P_{7.5}$ promoter (Fig. 1). The vaccinia virus recombinant expressing the TACV GPC protein (VV–GTac) contained nucleotides 17–67 of the 5′ non-coding sequence of the genomic S RNA plus the entire coding sequence of GPC at the 5′ end and 28 nucleotides of the intergenic region at the 3′ end. The recombinant expressing the TACV N protein (VV–N) included the entire N coding region surrounded at the 5′ end by the complete 5′ non-coding sequence of the S antigenome and, at the 3′ end, by 76 nucleotides of the intergenic region. The recombinant expressing the JUNV glycoprotein (VV–GJun) contained the entire 5′ non-coding sequence followed by the complete JUNV GPC coding sequence and 8 nucleotides of the intergenic region.

In order to characterize the TACV-specific proteins, CV-1 cells were infected with the vaccinia virus recombinants or with TACV, cells were pulse-labelled and proteins were immunoprecipitated with TACV-specific immune sera and resolved by gel electrophoresis. As seen in Fig. 2(A), the TACV GPC (molecular mass 69 kDa) and N (68 kDa) proteins migrated very closely when TACV-infected cell lysates were immunoprecipitated with hyperimmune serum to TACV (lane 3) and were identified with monospecific sera (lanes 2 and 4). Vaccinia virus-expressed TACV GPC and N proteins were recognized by hyperimmune serum to TACV and showed identical mobility in gel electrophoresis to that of authentic TACV proteins (lanes 1 and 6). TACV GPC is processed to the structural proteins G1 and G2, which co-migrate and are detected as a diffuse band of between 35 and 39 kDa (Rossi *et al.*, 1996). In order to compare the processing of recombinant and authentic GPC, TACV- and VV–GTac-infected cells were labelled for 16 h prior to immunoprecipitation. Analysis of the proteins showed that GPC from both TACV- and VV–GTac-infected cells was processed similarly (Fig. 2B).

The expressed JUNV GPC was characterized by radioimmunoprecipitation of cell lysates from infections with the vaccinia virus recombinant or with JUNV (Fig. 2C). In JUNV infection, both the GPC (65 kDa) and N (60 kDa) proteins immunoprecipitated with hyperimmune serum to JUNV and with monoclonal antibodies specific to each protein (Fig. 2C; lanes 1–3). The antigenic similarity between the glycoproteins expressed by JUNV and vaccinia virus was demonstrated by the reactivity of the recombinant protein to hyperimmune serum to JUNV and to a pool of four monoclonal NAs to JUNV glycoprotein (Fig. 2C; lanes 4 and 5). It should be mentioned that cells infected with VV–GJun reacted with each of the monoclonal antibodies, as detected by immunofluorescence (not shown). The recombinant and authentic

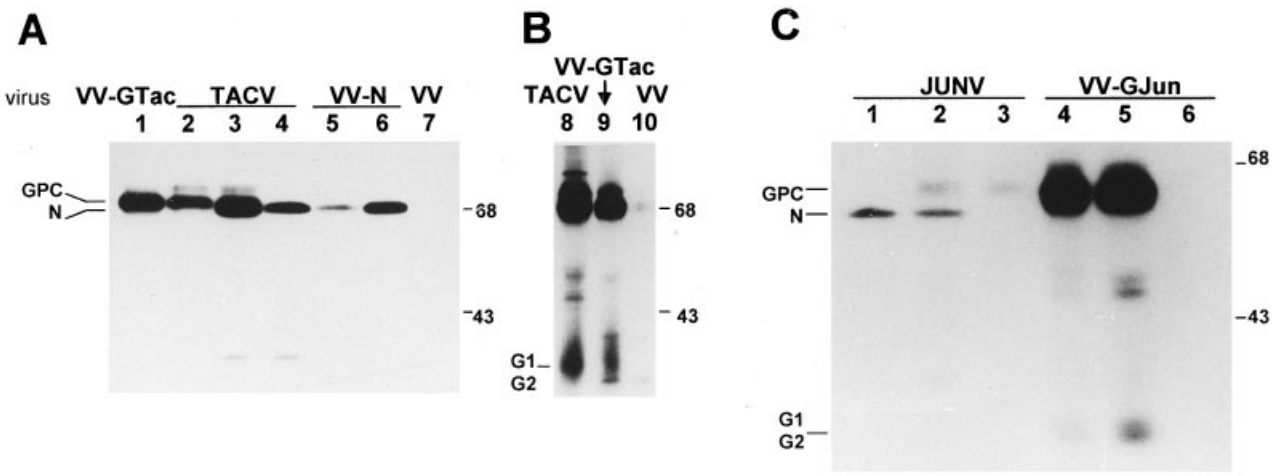

Fig. 2. PAGE of radiolabelled proteins immunoprecipitated from infected cell lysates. (A) CV-1 cells were infected with TACV (m.o.i. = 1) for 24 h or with VV–N, VV–GTac or VV (m.o.i. = 3) for 16 h. At these times post-infection, cell monolayers were labelled for 1 h, cell lysates were prepared and proteins were immunoprecipitated with specific immune sera as indicated in Methods. Proteins from TACV-infected cells were immunoprecipitated with hyperimmune serum to TACV (lane 3) or with monospecific sera (Rossi *et al.*, 1996) to TACV GPC (lane 2) or N (lane 4). Cells infected with VV–GTac (lane 1), VV–N (lane 6) or VV (lane 7) were immunoprecipitated with hyperimmune serum to TACV. Cells infected with VV–N were also immunoprecipitated with monospecific serum to TACV N (lane 5). Film was exposed for 20 h. (B) Cells infected as indicated in (A) were labelled for 16 h, starting at 10 h post-infection with TACV (lane 8) or at 6 h post-infection with VV–GTac (lane 9) and VV (lane 10). Cell lysates were immunoprecipitated with hyperimmune serum to TACV. Film was exposed for 48 h. (C) CV-1 cells were infected with JUNV (m.o.i. = 0·1) for 48 h (lanes 1–3) or with VV–GJun (m.o.i. = 3) for 16 h (lanes 4–6). At these times post-infection, cells were labelled for 4 h and polypeptides from cell lysates were immunoprecipitated with a pool of monoclonal antibodies to N (lanes 1 and 6), hyperimmune serum to JUNV (lanes 2 and 5) or a pool of four monoclonal antibodies to JUNV glycoproteins (lanes 3 and 4). Film was exposed for 48 h. In all cases, proteins were immunoprecipitated from about 1×10^5 cells. Numbers at the right correspond to the positions of the molecular mass markers (in kDa).

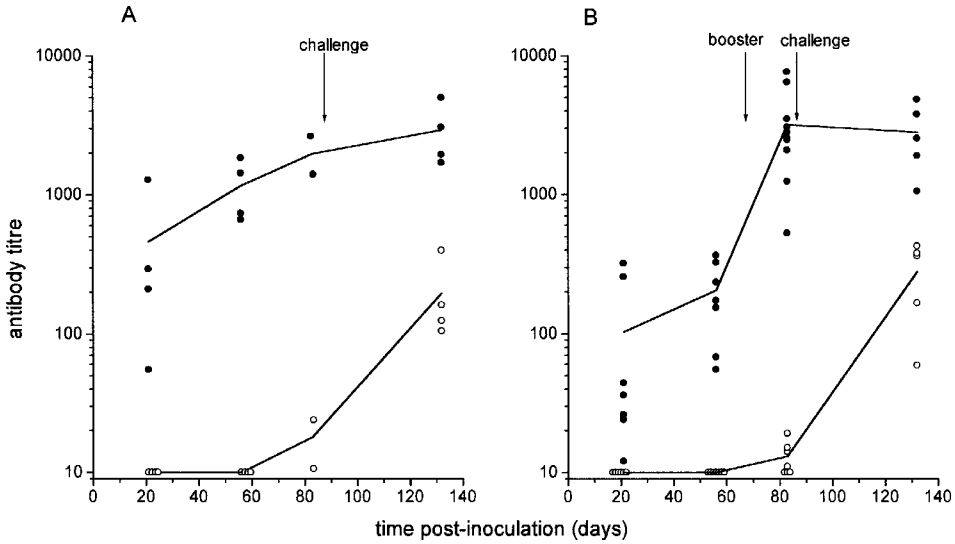

Fig. 3. NA to TACV and JUNV in guinea pigs inoculated with VV–GTac or with TACV. Guinea pigs were inoculated with TACV (four animals) (A) or with VV–GTac (10 animals) (B) as indicated in Methods. Animals inoculated with VV–GTac received a second inoculation (booster) 68 days after initial immunization. Mean NA titres to vaccinia virus increased from 150 at day 59 to 2640 at day 82 (not shown). All animals were challenged with JUNV (1×10^3 p.f.u.) 87 days after the single inoculation with TACV or the initial inoculation with VV–GTac. NA to TACV (●) and to JUNV (○) were analysed at the times indicated. Only two of the animals vaccinated with TACV were bled at day 82 after inoculation. Mean titres are connected by lines.

JUNV GPC migrated similarly in gel electrophoresis. The higher level of GPC expression by vaccinia virus compared with JUNV allowed detection of the processed glycoproteins (33–38 kDa) in cells infected with the recombinant.

Immunogenicity of the expressed TACV proteins

The immunogenicity of vaccinia virus-expressed TACV proteins was assessed in guinea pigs. Animals were divided

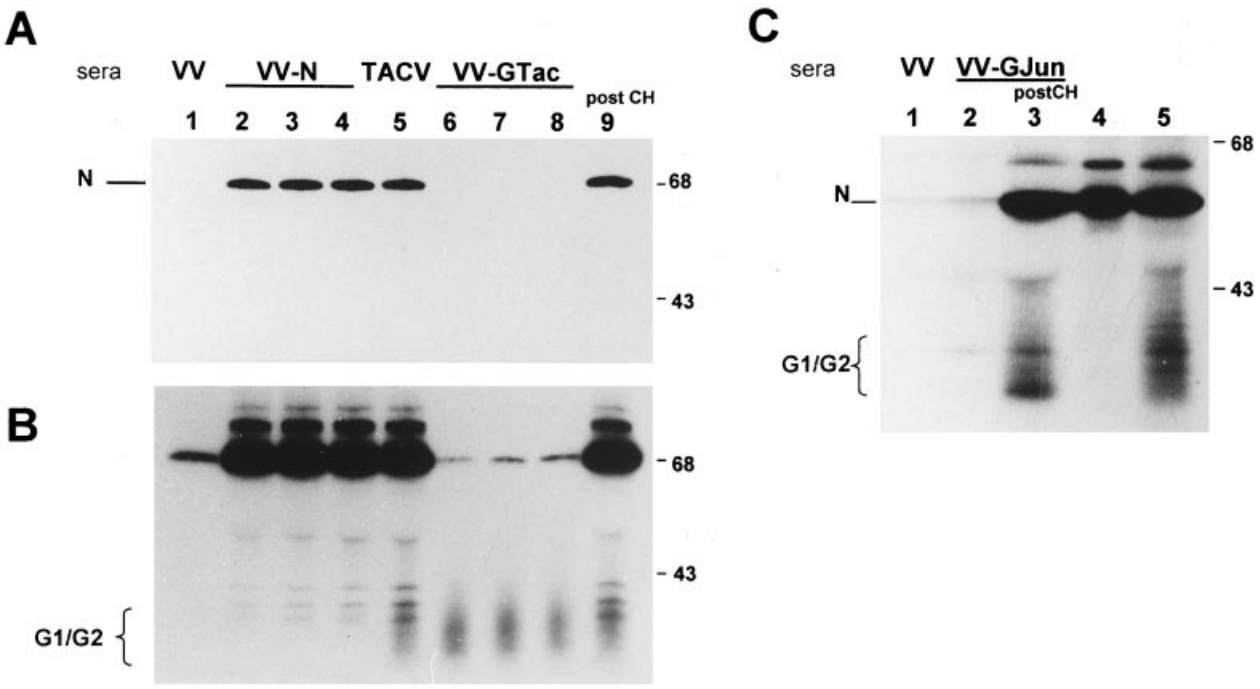

Fig. 4. Immunoprecipitation of authentic viral proteins with sera from guinea pigs immunized with recombinant vaccinia viruses. (A)–(B) Four groups of guinea pigs were immunized with VV–GTac (10 animals), VV–N (six animals), VV (six animals) or TACV (four animals) as indicated in Methods. At day 59 after the single immunization with TACV or at day 82 after primary inoculation with the vaccinia virus recombinants or VV, animals were bled and sera were pooled and evaluated for the ability to immunoprecipitate proteins from labelled TACV particles. Sera were pooled as follows: all animals immunized with VV (lane 1) or with TACV (lane 5); sera from two animals each inoculated with VV–N (lanes 2–4); sera from two (lane 6), three (lane 7) and five (lane 8) animals inoculated with VV–GTac. The latter sera corresponded to VV–GTac-immunized animals that later survived challenge with JUNV. Lane 9: immunoprecipitation performed with post-challenge (post CH) sera pooled from surviving VV–GTac-immunized guinea pigs, collected 45 days after challenge. All sera were tested at 1 : 50 dilution. In (A), the gel was exposed for 5 h to detect N protein. In (B), the same gel was exposed for 40 h to detect G1/G2 proteins. The distinct bands migrating as 38–42 kDa proteins that appeared in the over-exposed film when sera contained antibodies to N (B, lanes 2–5 and 9) correspond to degraded N in the TACV particle preparation. (C) Two groups of guinea pigs were doubly vaccinated with VV–GJun (18 animals) or with VV (six animals) and challenged with JUNV as indicated in Methods. Pre-challenge sera were collected 82 days after initial inoculation and post-challenge sera (post CH) at 32 days after challenge. Sera were pooled and evaluated for the ability to immunoprecipitate JUNV polypeptides from supernatant medium of JUNV-infected cells labelled for 16 h. Lanes: 1, pooled pre-challenge sera from all animals vaccinated with VV; 2, pooled pre-challenge sera from the 13 VV–GJun-vaccinated animals that later survived JUNV challenge; 3, pooled post-challenge sera from the 13 surviving VV–GJun-inoculated animals collected 32 days after challenge. Sera were tested at 1 : 15 dilution. Lanes 4 and 5 correspond to immunoprecipitations performed, respectively, with a pool of two monoclonal antibodies to JUNV N or with hyperimmune serum to JUNV. Film was exposed for 3 days. Numbers at the right correspond to the positions of molecular mass markers (in kDa).

into four groups and were immunized with 10^8 p.f.u. of either VV–N (six animals), VV–GTac (10 animals) or VV (six animals) or with 10^5 p.f.u. TACV (four animals). At 21 and 59 days after infection, all animals that had been immunized with VV or with the vaccinia virus recombinants displayed low but reproducible NA titres to vaccinia virus, indicating that they had all been successfully infected (not shown). At these times post-infection, all animals immunized with VV–GTac exhibited NA to TACV, although titres were lower than in TACV infections. None of the animals immunized with TACV or with VV–GTac elicited NA to JUNV (Fig. 3). As expected, none of the animals vaccinated with VV–N developed NA to TACV (not shown).

At 68 days after the initial immunization, animals that had been inoculated with either VV–N, VV–GTac or VV received a second immunization with the corresponding virus. Sera taken 14 days later (82 days after initial immunization) revealed that NA titres to vaccinia virus had increased approximately 20-fold compared with pre-booster sera. NA titres to TACV in animals that had been boosted with VV–GTac increased in parallel, displaying post-booster titres comparable to those generated in TACV infection (Fig. 3). In some of the animals inoculated with VV–GTac and TACV, a low but reproducible level of NA to JUNV was detected. This last result is in agreement with previous observations that, in guinea pigs infected with TACV, cross-reacting NA to JUNV appeared around 60 days post-infection (Martinez Peralta *et al.*, 1993).

The immunogenicity of the recombinant proteins after secondary immunization was also assessed by immunoprecipitation, with labelled TACV particles as antigen. Analysis was performed with pooled sera, which reflected results

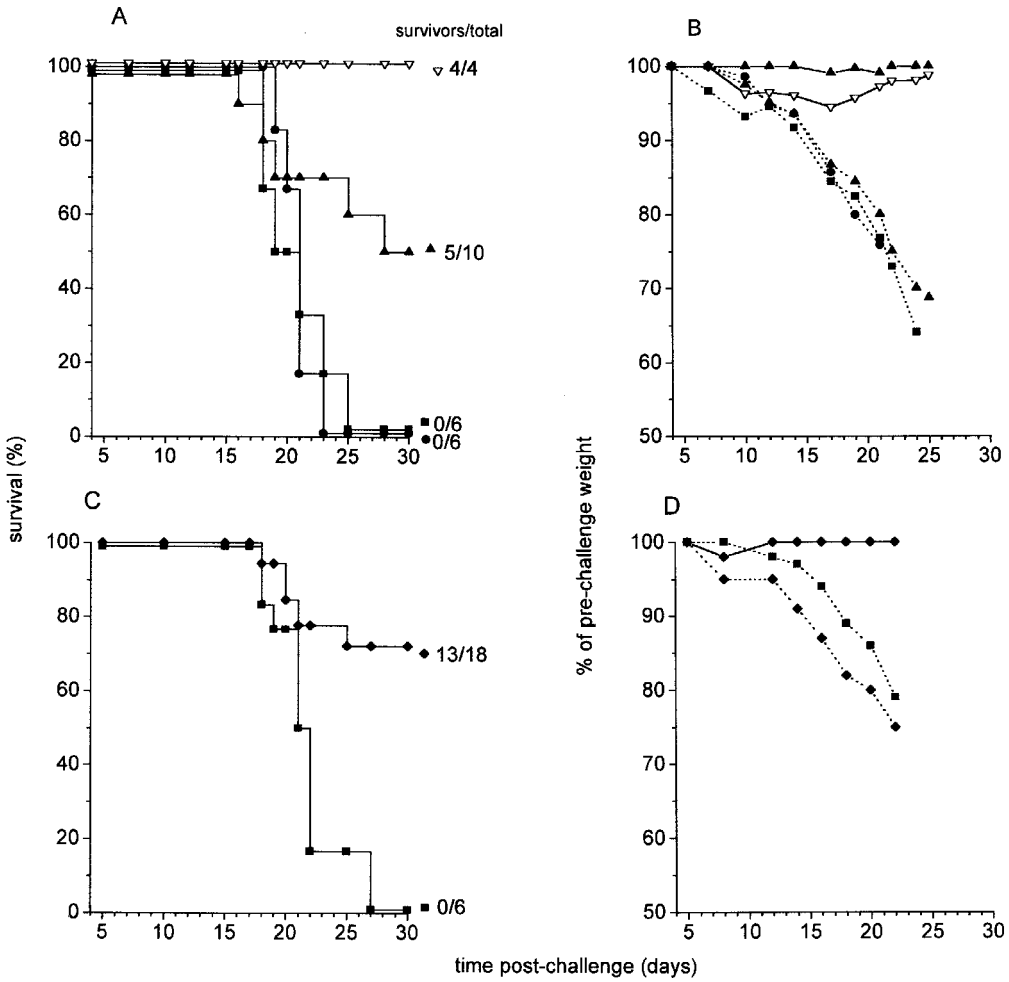

Fig. 5. Guinea pig survival and variation in body weight following challenge with JUNV. Immunization and challenge of guinea pigs were performed as indicated in Methods. (A)–(B) Immunization with TACV (▽), VV–GTac (▲), VV–N (●) or VV (■). (C)–(D) Immunization with VV–GJun (◆) or VV (■). Challenge with JUNV was performed 87 days after initial immunization. Mortality (A and C) and weight of animals that survived (solid lines) or died (dotted lines) (B and D) are shown. A and B and C and D represent independent experiments. Significant differences were detected between both VV and VV–N and VV–GTac and also between VV and VV–GJun (χ^2, $P < 0{\cdot}01$).

obtained with individual samples. Animals inoculated with VV–N or TACV displayed antibodies to N (Fig. 4A, lanes 2–5). Those inoculated with VV–GTac or TACV elicited antibodies to the processed glycoproteins G1/G2. The latter were detected after longer exposure of the film (Fig. 4B, lanes 5–8). Each of the recombinant viruses appeared to generate amounts of antibody comparable to the TACV infection.

Heterologous protection of guinea pigs from lethal challenge with JUNV

In order to evaluate the ability of recombinant vaccinia viruses expressing TACV proteins to protect against experimental AHF, we used the guinea pig model under conditions (JUNV strain and dose) that led to death of all unprotected animals. Guinea pigs immunized only once with TACV or twice with recombinant vaccinia viruses were challenged, 87 days after initial immunization, with 1×10^3 p.f.u. of the XJ strain of JUNV. As a control, six animals vaccinated twice with VV were also challenged. Results are shown in Fig. 5(A, B). All animals immunized with VV developed clinical signs typical of experimental AHF, with weight loss starting at about 7 days after challenge and death with haemorrhagic manifestations at around day 20 [mean time to death (MTD) $20{\cdot}7 \pm 2{\cdot}6$ days]. Immunization with VV–N did not afford protection and animals died (MTD $20{\cdot}8 \pm 1{\cdot}2$ days) with clinical signs identical to those in the VV-immunized controls. As reported previously (Damonte *et al.*, 1978; Coto *et al.*, 1980), immunization with TACV provided 100% protection and animals exhibited no clinical signs of JUNV infection. Of the 10 animals inoculated with VV–GTac, five survived, showing no signs of experimental AHF. Those guinea pigs that died after JUNV challenge (MTD $21{\cdot}2 \pm 4{\cdot}5$ days) showed clinical signs and loss of body weight comparable

to animals from other unprotected groups, but autopsy revealed less-severe haemorrhagic lesions.

The specific humoral immune response in animals that survived JUNV infection was assessed by plaque-reduction neutralization assays to TACV and JUNV. The level of NA to TACV was maintained, whereas a secondary immune response to JUNV was established in both TACV- and VV–GTac-immunized animals, with an increase in the levels of serum NA against JUNV from undetectable or low values before challenge (10–20) to titres ranging from 59 to 424 after challenge (Fig. 3). In VV–GTac-inoculated guinea pigs, there was no direct relationship between the presence or absence of NA to JUNV before challenge and the survival or death of the animals after JUNV infection.

We also analysed whether VV–GTac-inoculated animals that survived JUNV challenge generated specific antibodies to JUNV N protein. To this end, labelled TACV particles were used as antigen for immunoprecipitation, as it is known that antibodies to JUNV N recognize TACV N (Damonte *et al.*, 1986; Sanchez *et al.*, 1989). Analysis of the proteins by gel electrophoresis showed that animals inoculated with VV–GTac that had antibodies only to TACV glycoproteins prior to challenge elicited antibodies to N after challenge (Fig. 4 A and B, lanes 8 and 9).

Protection of guinea pigs against experimental AHF by vaccinia virus recombinants expressing JUNV glycoproteins

Having established that vaccinia virus expressing TACV glycoprotein but not N induced protection against JUNV challenge, we studied the capacity of vaccinia virus expressing JUNV glycoprotein (VV–GJun) to provide protection against experimental AHF. For immunization with VV–GJun, we followed a protocol identical to that used previously with vaccinia virus expressing TACV antigens, as this has proved to induce levels of antibodies comparable to those in infection by the virus. Two groups of animals were vaccinated with 1×10^8 p.f.u. of either VV–GJun (18 animals) or VV (six animals) and, 68 days after initial inoculation, animals were boosted with the same doses of the corresponding virus. To demonstrate a serological response to immunization, animals were bled at day 82 after primary inoculation and NA titres to vaccinia virus and to JUNV were determined (Table 1). Titres of antibodies to vaccinia virus revealed that all animals had been successfully infected, with values ranging from 1200 to 5000. The mean titre to vaccinia virus of 2490 after two immunizations with VV–GJun compared well with that of 2640 after double immunization with VV–GTac (see legend to Fig. 3). Titres of NA to JUNV were low (values ranged from 13 to 250) or undetectable (four animals of 18). Antibodies specific to JUNV in pre-challenge sera could be detected by immunofluorescence (not shown), but attempts to immunoprecipitate radiolabelled JUNV glycoproteins were unsuccessful (Fig. 4C, lane 2).

At day 87 after initial immunization with VV–GJun or VV, animals were challenged with lethal doses of JUNV. All animals inoculated with VV died, whereas 13 of the 18 animals inoculated with VV–GJun survived (72%). No symptoms of experimental AHF were observed in the protected animals (Fig. 5). Those VV–GJun-inoculated animals that did not survive JUNV challenge (MTD $22 \pm 2{\cdot}3$ days) developed symptoms of disease similar to the vaccinia-inoculated controls, although at death, haemorrhagic lesions were less severe. Protection did not correlate with the pre-challenge level of NA to JUNV. Surviving animals developed a significant antibody response to JUNV proteins, as shown by the 20-fold increase of NA titres to JUNV compared with pre-challenge sera (Table 1) and by the acquired ability to immunoprecipitate radiolabelled JUNV glycoproteins and nucleoprotein (Fig. 4C, lane 3).

Table 1. Immune responses of guinea pigs to vaccinia virus expressing JUNV glycoproteins and to challenge with JUNV

Mean NA titres are shown. Two groups of guinea pigs were immunized with 1×10^8 p.f.u. VV (six animals) or VV–GJun (18 animals) at days 0 and 68. At day 82 after initial inoculation, animals were bled and NA titres to VV and JUNV were determined (Pre-challenge sera). Challenge with lethal doses of JUNV was performed on day 87 after initial inoculation. Sera from the VV–GJun-inoculated animals that survived JUNV infection ($n = 13$) were collected 32 days after challenge and NA titres to JUNV were determined (Post-challenge sera). ND, Not done.

	Pre-challenge sera		Post-challenge sera
Immunogen	VV	JUNV	JUNV
VV–GJun	2490	71	1600
VV	3200	< 10	ND

Discussion

The initial aim of the present report was to define the nature of the TACV antigens involved in cross-protection against JUNV challenge. To this end, we constructed recombinant vaccinia viruses expressing each of the two main antigenic TACV proteins, the glycoprotein and the nucleoprotein. The expressed proteins were characterized, showing that they resembled authentic TACV proteins closely, and the capacity of the recombinant viruses to induce antibodies in guinea pigs was studied. Priming and boosting with VV–GTac or with VV–N induced levels of antibodies reactive to TACV proteins detected by neutralization and immunoprecipitation comparable to those elicited in TACV infection, demonstrating the efficacy of VV–GTac and VV–N expression and immunogenicity.

In order to evaluate the ability of the recombinant viruses to protect against experimental AHF, we used the guinea pig

model under conditions that led to death of all unprotected animals. Of the two recombinant vaccinia viruses tested, that which expressed TACV GPC conferred protection upon 50% of the animals. The recombinant expressing TACV N failed to afford protection.

The level of protection afforded by vaccinia virus expressing TACV GPC is less than the 100% protection found when TACV is used as vaccine. Full protection by whole virus might reflect the optimum presentation of antigens in the virus particle and/or the requirement for other viral proteins. However, at least for LASV, simultaneous inoculation of vaccinia virus recombinants expressing GPC or N has been shown to be less protective than single immunization with each recombinant virus (Morrison *et al*., 1989). It should be remarked that correct presentation of viral antigens seems critical for protection against diseases caused by arenaviruses. This point is highlighted by the success of live-virus vaccines such as TACV and Mopeia virus for protection against JUNV and LASV infections and the failure of killed-virus vaccines (Clegg, 1992). On the other hand, the protective role played by viral proteins encoded in the L RNA segment should not be overlooked. The p11/Z protein, for instance, has been found in equimolar proportions with G1/G2 in TACV and LCMV particles and might be associated with N and G2 (Salvato, 1993; Rossi *et al*., 1996).

Immunization of guinea pigs with recombinant vaccinia virus expressing the glycoproteins of TACV greatly increased the possibility of survival of experimental AHF in this animal model. Animals immunized with VV–GTac generated high levels of NA to TACV but undetectable or very low levels of NA to JUNV. After challenge, the surviving animals developed a strong antibody response to JUNV glycoproteins, as detected by the appearance/increase of NA to JUNV and to JUNV nucleoprotein as assayed by immunoprecipitation. This indicated that guinea pigs survived after an actual infection by the challenge virus.

Several lines of evidence support the notion that, at variance with the Old World arenaviruses, NA can play a role in protection against infection with New World arenaviruses (Peters *et al*., 1987; Weissenbacher *et al*., 1987). It might therefore be expected that generation of NA to JUNV by immunization with the homologous glycoprotein should reduce replication of the challenge virus initially, leading to improved protection in comparison with the heterologous TACV glycoprotein. We therefore constructed a recombinant vaccinia virus that expressed the JUNV glycoprotein (VV–GJun). Recombinant JUNV glycoprotein was found to resemble authentic JUNV glycoprotein closely, retaining antigenic sites defined by monoclonal NA to JUNV.

Guinea pigs were next immunized with VV–GJun by using a protocol identical to that used previously for inoculation with vaccinia virus expressing TACV antigens. When sera collected 82 days after primary inoculation with VV–GJun were analysed, a clear difference was noted in antibody response between the guinea pigs immunized with VV–GJun and those previously inoculated with VV–GTac, as detected both by homologous NA titres (Table 1 and Fig. 3) and by immunoprecipitation of the corresponding glycoprotein (Fig. 4).

We have as yet no explanation for the low level of antibodies directed to JUNV glycoproteins in guinea pigs immunized with VV–GJun compared with the high efficacy of VV–GTac in generating antibodies to TACV glycoproteins. The same vaccinia virus strain and similar constructs were used for generation of each recombinant and the levels of glycoprotein expression were comparable, as detected by immunoprecipitation of lysates from cells infected with VV–GJun or VV–GTac. Furthermore, infection of guinea pigs with vaccinia virus that expressed either JUNV or TACV glycoproteins was equally successful, as indicated the NA titres to vaccinia virus in each experiment (Table 1 and legend to Fig. 3). In addition, special attention was devoted to ensuring that the gene sequence of the JUNV GPC molecular clone inserted in vaccinia virus corresponded exactly to that of the JUNV XJ strain used for challenge and in neutralization tests (see Methods). The capacity of VV–GJun to generate antibodies to JUNV glycoproteins in guinea pigs could not be compared with that of JUNV XJ strain as animals died before eliciting detectable NA (Weissenbacher *et al*., 1987; our own unpublished observations).

Although VV–GJun, contrary to our expectations, failed to generate high levels of NA to JUNV, immunization of guinea pigs with this recombinant virus protected 13 of 18 animals against lethal JUNV challenge. The surviving animals, however, were susceptible to JUNV replication, as antibodies to JUNV N protein (not encoded in the recombinant) were detected after challenge. Whether animals immunized with the homologous or the heterologous glycoproteins survived or died appeared to be defined early after challenge, as clinical signs and loss of body weight started at days 7–9 and death occurred at about day 20, as in the VV-inoculated controls. Meanwhile, those animals that survived JUNV challenge showed none of the symptoms of experimental AHF.

As in vaccination with the heterologous glycoprotein, animals protected with VV–GJun developed a significant increase of NA titres to JUNV. It might be that rapid generation of NA due to a B cell-memory response in guinea pigs inoculated with VV–GJun or VV–GTac provides the critical protective component and/or that a cell-mediated response is involved in protection. The mechanism(s) underlying protection requires further research; however, our data indicate that, whatever the mechanism(s), vaccinia virus recombinants expressing the glycoprotein, whether homologous or heterologous, can protect guinea pigs against lethal JUNV infection.

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