# INTRODUCTION OF RESISTANCE TO BOVINE ROTAVIRUS BY GENE TRANSFER

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## SUMMARY

Experimentation in cell culture and in laboratory mice was designed to test receptor blocking as a potential mechanism of resistance to diarrheas in newborn livestock. Expression constructs containing the bovine rotavirus (BRV) outer shell protein (VP4 or VP7) DNAs were transfected into susceptible cells in culture or microinjected into mouse embryos. Four of the cell lines transfected with VP4 or VP7 produced the VP mRNA and showed increased resistance to BRV. Microinjection and transfer of over 2,300 embryos resulted in four lines of mice carrying the stably inherited VP7 transgenes, but none with VP4.

## INTRODUCTION

Rotaviruses are a major etiological agent of diarrhea in neonate mammals and are responsible for large losses of domestic animals - globally $ 2 billion annually in cattle alone - and deaths of human infants. Two rotaviral outer shell (capsid) proteins, VP4 (Fukudome et al., 1989; Bass et al., 1991) and VP7 (Sabara et al., 1985; Fukuhara et al., 1988), have been implicated in receptor-mediated infection.

At present, it is necessary to vaccinate the dam and rely on maternal antibodies - passive immunity - to protect the neonate against BRV. Therefore, exploration of non-immunological resistance mechanisms appeared worthwhile. To induce genetic resistance to BRV, we have attempted to interfere with the mechanism of rotaviral attachment and entry by genetically manipulating the host cells. This is the first report of results from the project.

## MATERIALS AND METHODS

**Expression plasmids**: The VP7 and VP4 cDNAs were inserted into the BamHI site of pSG5 (Stratagene), a commercial eukaryotic expression vector containing the early SV40 promoter and polyadenylation signal, as well as intron II of the rabbit beta-globin gene. The resulting constructs containing the VP7 or VP4 cDNA inserts were designated VO1 and VO2, respectively. Construct VO3 was the linearized DNA derivative of VO1 from which the pSG5 was removed, and VO4 represented VP4 cDNA inserted in the pTX1 (Strategene) vector that contained a retroviral long terminal repeat, a thymidine kinase promoter, and a neomycin resistance gene.

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**Cell culture experiments:** The MA104 African green monkey embryonic kidney cell line, known to be susceptible to rotaviruses, was obtained from the American Type Culture Collection. BRV was isolate C486 (Babiuk et al., 1977), a group A, serotype 6 virus. It was propagated in MA104 cells with trypsin activation according to standard procedures.

The constructs VO1 and VO2 were each co-transfected separately with pSV2neo (Southern and Berg, 1982), to confer resistance to G418, into MA104 cells by the calcium phosphate technique (Graham and Van der Eb, 1973). Selected transfected cell colonies were harvested, expanded and frozen.

Transfected cells in culture harbouring the DNA constructs were identified with a polymerase chain reaction (PCR). Oligonucleotide primers for the amplification of portions of the VP cDNA inserts were synthesized on Applied Biosystems Synthesizer model 392. RNA for Northern blot analyses was extracted from four to eight million cells per transfected cell line. Probes used were the BamHI fragments from VO1 (1062 bp) or VO2 (2364 bp), labelled to high specific activity with $^{32}P$ using the hexamer priming method of Feinberg and Vogelstein (1983). Approximately 1 $\mu$g total RNA per clone was analyzed for the presence of polyadenylated RNA using the Gene-amp reverse-transcriptase PCR (RT-PCR) method (Perkin Elmer Cetus) using the oligo-d(T) for the reverse- transcriptase reaction, as well as the primers described above. For Southern blot analyses, genomic DNA was extracted from frozen cell samples. Indirect immunofluorescence tests on the transfected cells were performed with a primary polyclonal rabbit anti-BRV antibody. Non-denatured cellular proteins were isolated from transfected cells for Western blot analyses after three freeze-thaw cycles. Bands were visualized using the BCIP/NBT colour development system.

For rotavirus challenge tests, plaque assays (Aha and Sabara, 1990) were run using cultures of the MA104 cells (approximately .5 million cells per well) expressing either VP4 or VP7 RNA. The cells were exposed to ten-fold serial dilutions of trypsinized BRV to determine differences in rotaviral susceptibility.

**Mouse experiments:** The linearized VO1, VO2, VO3, and VO4 DNA constructs were microinjected into the pronuclei of one-cell embryos from the ICR strain using standard protocols (Gordon et al. 1980) and transferred to recipient females previously mated to vasectomized males. Transgenic progeny were detected using the PCR assay and confirmed by Southern blots. The VP mRNA in several tissues, including the intestine was assayed by the RT-PCR method. Protocols were similar to those described above for cells in culture. The transgenic mice obtained were mated to mice from the same strain and the semicongenic transgenic lines so formed were further maintained for three generations by mating hemizygote males to females without the transgene. The status of the selection candidates was examined by PCR reactions. Besides maintaining the lines described above, the transgenes were also introgressed into the randombred Cp strain (Nagai et al. 1985) to test the effect of the transgenes in two different genomic backgrounds.

## RESULTS

**Cell culture experiments:** The transfection of the MA104 cells resulted in 12 clones with stably integrated VO1 and six with VO2. Based on Northern blot analysis of total RNA, three VO1 clones (VO1.5, VO1.8, VO1.12) and two VO2 clones (VO2.19 and VO2.21) were found to express transgene-specific mRNA. Indirect immunofluorescence of transfected MA104 cell lines with a polyclonal antibody to BRV showed no rotavirus-specific staining patterns. Similarly, in Western blot analyses, none of the clones expressing transgene mRNA were found to contain detectable levels of protein. However, the results of rotaviral challenge indicated that viral resistance exists in the transfected cell lines VO1.5, VO1.8, VO2.19, and VO2.21 compared to the untransfected MA104 cell line. An example of challenge

results obtained in most tests is in Table 1. One clone carrying the VP7 transgene, VO1.12, showed no significant difference in susceptibility to BRV.

Table 1 Reduction in susceptibility of MA104 cells transfected with the DNA constructs containing the VP4 or VP7 cDNA

| | Cell type or clone number | | | | | |
|---|---|---|---|---|---|---|
| | MA104 | VO1.5 VP7 | VO1.8 VP7 | VO1.12 VP7 | VO2.19 VP4 | VO2.21 VP4 |
| Ave. No. of plaques | 105 | 4** | 21** | 42 | 7** | 9** |
| Reduction (%) | | 96.2 | 80.0 | 60.0 | 93.3 | 91.4 |

** $P < 0.01$

**Mouse experiments:** The efficiency of producing transgenic mice was low - less than 3 % of pups born and less than .5 % of over 2,300 microinjected and transferred embryos. The transgenes in genomic DNA were detected by slot blots or PCR and confirmed by Southern blots. Four lines of mice carrying the VP7 transgene were formed. Each line originated from a single individual born from the microinjected embryos. In the lines, the transgenes have been transmitted in a Mendelian fashion. The VP7 mRNA was detected in the lines by RT-PCR. Attempts to detect the VP7 protein have been, so far, unsuccessful. The lines are being tested for the effect of the transgenes on reproduction of parents and survival of progeny.

The attempts to produce transgenic lines from DNA constructs containing the VP4 cDNA were unsuccessful.

## DISCUSSION

In cell culture, the VP7-containing constructs became stably integrated in the susceptible MA104 cells at a higher rate than did the VP4-containing constructs. This observation is in agreement with the lack of success in producing transgenic mice from the constructs with VP4. The difference in the integration rate of VP7 and VP4 in cell culture may result from the larger size of the VO2 construct compared to the VO1 construct, or from detrimental effects of the VP4 DNA inserts or of the protein on viability of some cells. Our observations favour the latter explanation. Similar reasons may have caused the difference in producing transgenic mice.

No VP4 or VP7 proteins were detected by Western blots or indirect immunofluorescence staining. This may indicate that there is no translation of the transgene mRNAs, that the proteins are synthesized but are immediately degraded, that the proteins are synthesized but take on a different conformation such that they are not detectable with the antibodies used, or that the proteins are synthesized and secreted, thus leaving an undetectable level present within the cells. It is also possible that the antibodies used were not functioning as expected.

The findings that the transformed cells produced fewer plaques in most viral challenge assays are considered preliminary. Significantly fewer plaques formed with two clones expressing the VP4 transgene (VO2.19 and VO2.21) and two expressing the VP7 transgene (VO1.5 and VO1.8) than in the

original MA104 cells. These results suggest that there was interference at some level with the rotaviral infectious cycle in the transfected cells. The mechanism of the interference is not clear. The transgene products may interfere with binding or with viral assembly. Alternatively, the transgene RNA may interfere with transcription or translation of viral RNA. Further work will be required to explain the reduced susceptibility to infection associated with the transgenes.

An example of a naturally occurring inherent resistance to the avian leukosis virus (ALV) involving cell receptors exists in chickens (Crittenden, 1975). ALV-resistant chickens (Salter and Crittenden, 1989) were obtained by transfer to the host of a gene that expresses the ALV envelope proteins. Our results from the cell culture experiments confirm that introduction of viral genetic information into the host genome is a potential virus resistance mechanism that can be induced in animals by gene transfer. The results are encouraging because at least one of the two BRV capsid proteins stably integrated in the mouse genome. More work is needed to explain the lack of success in producing mice with the VP4 transgene.

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