Reports

Cloned Viral Protein Vaccine for Foot-and-Mouth Disease: Responses in Cattle and Swine

Abstract. *A DNA sequence coding for the immunogenic capsid protein VP_3 of foot-and-mouth disease virus A_{12}, prepared from the virion RNA, was ligated to a plasmid designed to express a chimeric protein from the* Escherichia coli *tryptophan promoter-operator system. When* Escherichia coli *transformed with this plasmid was grown in tryptophan-depleted media, approximately 17 percent of the total cellular protein was found to be an insoluble and stable chimeric protein. The purified chimeric protein competed equally on a molar basis with VP_3 for specific antibodies to foot-and-mouth disease virus. When inoculated into six cattle and two swine, this protein elicited high levels of neutralizing antibody and protection against challenge with foot-and-mouth disease virus.*

Foot-and-mouth disease (FMD) is an acute and highly contagious febrile disease afflicting primarily cloven-hoofed animals. Although mortality seldom exceeds 5 percent in adult animals, it can reach 50 percent in young cattle and swine as a result of myocarditis. The infection often causes deformities of the feet, abortion, and mastitis.

The causative agent, FMD virus (FMDV), is a picornavirus of the genus Aphthoviridae. There are several viral serotypes—A, O, and C; Southern African Territories (SAT) 1, 2, 3; and Asia 1—and 65 or more subtypes. The virus has a molecular size of about 7×10^6 daltons and contains a plus-stranded RNA genome of approximately 8000 nucleotides (*1, 2*).

The disease is primarily controlled through eradication programs involving the slaughter and sanitary disposal of herds containing infected and exposed animals, or through intensive vaccination programs. Vaccines are produced primarily in the country of use by inactivation of virus grown in baby hamster kidney cells or bovine tongue epithelium (attenuated virus has been used as well). Outbreaks of FMD are frequently linked to incompletely inactivated vaccine or escape of virus from research and production facilities. The whole-virus vaccine preparations are unstable and require refrigeration.

We now report the biosynthesis in *Escherichia coli* of a safe, stable, and effective polypeptide vaccine for FMD.

Picornavirus proteins are synthesized in infected cells as a precursor polyprotein that is subsequently processed by cellular and virus coded proteases (*3*) into four major capsid proteins (VP_1, VP_2, VP_3, and VP_4) and numerous noncapsid proteins. Three of the four major capsid proteins have been extensively studied, and the amino terminal (*4*) and carboxyl terminal (*5*) amino acid sequences have been determined. The VP_3 protein (*6*) when used to inoculate swine elicits a neutralizing antibody response (*7*), and protects both swine and cattle from infection (*8, 9*). Likewise, a 13-kilodalton (kd) peptide derived from VP_3 by CNBr cleavage was immunogenic (*4, 9*). On the basis of these results and others indicating that the VP_3 gene is near the center of the FMDV genome (*10*), we constructed a series of plasmids containing subgenomic complementary DNA (cDNA) inserts (*11*), two of which are shown in Fig. 1. Restriction maps were determined for these and other overlapping cDNA inserts in order to accurately locate the VP_3 gene. Nucleotide sequence analysis of these inserts (*12, 13*) established the position of the VP_3 gene, because the deduced NH_2-terminal amino acid sequence (Fig. 2)

Table 1. Neutralizing antibody and immune responses of cattle and swine vaccinated with LE′-VP_3 fusion protein or VP_3 isolated from virus.

Subject	Antigen	Neutralizing antibody day after vaccination*								Challenge of immunity†		
		7	14	21	28	35	42	46	56	Mouth lesions	Foot lesions	VIA antibody‡
Cattle												
1	LE′-VP_3	<.3	.5	.3	1.2	2.3	2.7		2.9	0	0	−
2	LE′-VP_3	.2	.9	1.3	2.0	2.4	2.7		2.9	0	0	−
3	LE′-VP_3	.2	.4	1.1	1.7	1.9	2.1		2.9	0	0	−
4	LE′-VP_3	.2	.7	.7	1.1	2.2	2.6		2.9	0	0	−
5	LE′-VP_3	<.3	.9	1.0	1.1	2.6	2.5		2.6	0	+	−
6	LE′-VP_3	<.3	1.0	1.3	1.3	2.1	2.1		2.3	0	0	−
7	VP_3	.1	1.0	2.1	1.9	2.2	2.6		2.1	0	0	−
8	VP_3	<.3	.4	.4	.7	1.7	1.6		4.1	+++	+++	+
9	None†								3.4	+++	+++	+
10	None								3.5	+++	+++	+
11	None								3.3	+++	+++	+
12	None								3.3	+++	+++	+
Swine												
13	LE′-VP_3	<.3	<.3	.5	1.5			3.0		0	0	−
14	LE′-VP_3	<.3	.3	.6	1.4			3.0		0	0	−
15	None†							3.5		+++	+++	+
16	None							3.4		+++	+++	+

*Revaccinations on day 24 for swine and day 28 for cattle; titer is $-\log_{10}$ of serum dilution that protects 50 percent of suckling mice (*25*). The 46- and 56-day serums were collected 14 days after challenge. †Challenged on day 32 for swine and day 42 for cattle. Nonvaccinated animals 9 to 12 and 15 and 16 constituted the challenge groups; half were inoculated with virulent type A_{12}119ab virus and half were contact transmission controls; +, single small lesion, not generalized disease; +++, numerous lesions and generalized infection. ‡Presence, +, of virus-infection-associated antigen (VIA) antibody in serums collected 14 days after challenge indicated animal experienced FMD (*28*); −, VIA antibody absent.

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corresponds to that previously determined for the VP_3 protein (4, 9).

The coding sequence for nearly all of the VP_3 protein was recovered from the plasmid pT465 by cleavage with the restriction endonucleases Pst I and Pvu II (Figs. 1 and 2). Codons 8 to 211 of the VP_3 protein (Fig. 2) are within the 610 base pairs (bp) located between these two cleavage sites. This fragment was incorporated into plasmid pFM_1 with tryptophan (trp) promoter operator, designed to direct the synthesis of VP_3 linked to the *E. coli* protein trp ΔLE 1413 (14) which is coded from the trp leader (L) gene and from the last third of the trp E protein gene (Fig. 3). The LE segment, which has 190 amino acids (15), is extremely useful as part of a fusion protein because it is insoluble inside the bacteria and resistant to proteolytic degradation (16). The protein can be visualized as a refractile body in live bacteria by phase contrast microscopy and is recovered in the cellular debris of lysed *E. coli* (16). An Eco RI site was introduced into the trp LE gene at the trp E termination codon (15) to link the VP_3 gene in the correct reading frame to create the fusion protein LE′-VP_3. The expressed LE′-VP_3 fusion protein has a molecular size of about 44.4 kd. The VP_3 amino acids in LE′-VP_3 extend (Fig. 2) from serine at position 7 (part of the Pst I site) to glutamine at position 211, one amino acid short of a leucine which may be the COOH-terminus of VP_3 (17, 18).

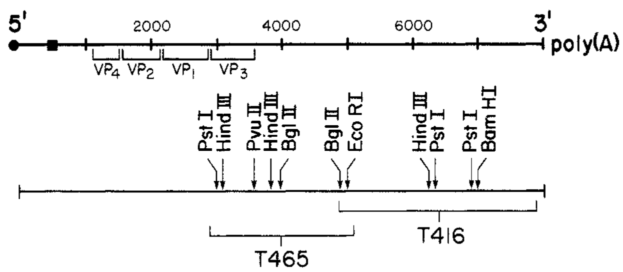

Fig. 1. The approximately 8000-nucleotide FMDV RNA genome is shown with its 5′ terminal VP_g protein (●), poly(C) tract near the 5′ end (■), and poly(A) at the 3′ end. Below the genome is a partial restriction map deduced from two cloned double-stranded cDNA molecules, T465 and T416, which span the lengths indicated. The cDNA molecules shown were synthesized with the use of an oligo(T) primer for reverse transcriptase, joined to the *E. coli* plasmid pBR322 with oligo(C) · oligo(G) tails, and cloned by standard methods (30). The RNA template FMDV A_{12} 119ab (4) was prepared by the method of Grubman *et al.* (31). The results of these cloning experiments are similar to those reported by Küpper *et al.* (17) and Boothroyd *et al.* (32).

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                    VP1                         1                   VP3                                 14
Leu Arg Leu Pro Ile Asp Pro Arg Ser Gln Thr Thr Ala Thr Gly Glu Ser Ala Asp Pro Val Thr Thr Thr
UUA CGC CUC CCG AUU GAC CCC CGG UCA CAA ACC ACC GCU ACC GGG GAG UCU GCA GAC CCU GUC ACC ACC ACC
                                                                    PstI
                                                                                         36     38
Val Glu Asn Tyr Gly Gly Glu Thr Gln Val Gln Arg Arg His His Thr Asp Val Ser Phe Ile Met Asp Arg
GUG GAG AAC UAC GGU GGU GAG ACA CAA GUC CAG AGA CGU CAC CAC ACG GAC GUC AGU UUC AUC AUG GAC AGA
                                                             54                                 62
Phe Val Lys Ile Lys Ser Leu Asn Pro Thr His Val Ile Asp Leu Met Gln Thr His Gln His Gly Leu Val
UUU GUG AAG AUA AAA AGC UUG AAC CCC ACA CAC GUC AUU GAC CUC AUG CAG ACC CAC CAA CAC GGG CUG GUG
                  HindIII
                                                                                                86
Gly Ala Leu Leu Arg Ala Ala Thr Tyr Tyr Phe Ser Asp Leu Glu Ile Val Val Arg His Asp Gly Asn Leu
GGU GCG UUG UUG CGU GCA GCC ACG UAC UAC UUC UCC GAC UUG GAG AUU GUU GUG CGG CAC GAU GGC AAU CUG

                                                                                               110
Thr Trp Val Pro Asn Gly Ala Pro Glu Ala Ala Leu Ser Asn Thr Gly Asn Pro Thr Ala Tyr Asn Lys Ala
ACC UGG GUG CCC AAC GGU GCC CCC GAG GCA GCC CUG UCA AAC ACC GGC AAC CCC ACU GCC UAC AAC AAG GCA

                                                                                               134
Pro Phe Thr Arg Leu Ala Leu Pro Tyr Thr Ala Pro His Arg Val Leu Ala Thr Val Tyr Asn Gly Thr Asn
CCG UUC ACG AGG CUU GCU CUC CCU UAC ACU GCG CCA CAC CGC GUG UUG GCA ACU GUG UAC AAC GGG ACG AAC

                                                                                               158
Lys Tyr Ser Ala Ser Gly Ser Gly Val Arg Gly Asp Phe Gly Ser Leu Ala Pro Arg Val Ala Arg Gln Leu
AAG UAC UCC GCG AGC GGU UCG GGA GUG CGA GGC GAU UUU GGG UCU CUC GCG CCG CGA GUC GCG AGA CAA CUU
                                                                                 179           182
Pro Ala Ser Phe Asn Tyr Gly Ala Ile Lys Ala Glu Thr Ile His Glu Leu Leu Val Arg Met Lys Arg Ala
CCU GCU UCU UUC AAC UAC GGU GCA AUU AAG GCC GAG ACC AUC CAC GAG CUU CUC GUG CGC AUG AAA CGG GCU

                                                                                               206
Glu Leu Tyr Cys Pro Arg Pro Leu Leu Ala Ile Glu Val Ser Ser Gln Asp Arg His Lys Gln Lys Ile Ile
GAG CUC UAC UGC CCC AGG CCA CUG CUG GCA AUA GAG GUG UCU UCG CAA GAC AGG CAC AAG CAG AAG AUC AUU
                    212                                                                        230
Ala Pro Gly Lys Gln Leu Asn Phe Asp Leu Leu Lys Leu Ala Gly Asp Val Glu Ser Asn Pro Arg Pro Phe
GCA CCC GGA AAA CAG CUG AAC UUU GAC UUA CUC AAG UUG GCA GGU GAC GUU GAG UCC AAC CCU AGA CCC UUC
                  PvuII
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Fig. 2. The nucleotide sequence, the derived amino acid sequence, and restriction map (Pst 1, Hind III, Pva II) of the VP_3 gene and contiguous areas from the plasmid pT465. The VP_3 gene begins at base 1 (NH_2-terminal threonine) and may terminate at codon 212 for COOH-terminal leucine. Abbreviations for the amino acid residues are: Ala, alanine; Arg, arginine; Asp, aspartic acid; Asn, asparagine; Cys, cysteine; Glu, glutamic acid; Gln, glutamine; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine.

The recombinant bacteria (*E. coli* 294/pFM_1) in 800 ml of culture grown at 37°C in a shaker flask (to $A_{550\ nm} = 2$) in tryptophan-depleted media (19) were collected by centrifugation at 4000*g*. The bacteria were resuspended in 80 ml of TEN [50 m*M* tris-HCl (*p*H 7.5), 0.5 m*M* EDTA, 0.3*M* NaCl], treated on ice for 15 minutes with lysozyme (1 mg/ml), and made 0.2 percent in NP-40. After 10 minutes, the sample was diluted with 120 ml of 1.5*M* NaCl and 12 m*M* $MgCl_2$ and stirred on ice with deoxyribonuclease (2 μg/ml, final concentration) for 1 hour. This treatment produced an *E. coli* lysate with insoluble LE′-VP_3 protein that was recovered by centrifugation at 4000*g* for 15 minutes at 4°C and then resuspended and washed three times with 30 ml of TEN. The precipitate contained 450 mg of protein by the BioRad dye-binding assay (about 45 percent of the total protein), of which 170 mg or approximately 17 percent of the total *E. coli* protein was the LE′-VP_3 fusion protein by PAGE (polyacrylamide gel electrophoresis) analytical scanning (20) (Fig. 4).

The fusion protein was purified by two successive sodium dodecyl sulfate (SDS)–PAGE runs using a 1-cm-thick, 10 percent slab gel in 8*M* urea (21). The protein-containing bands, which were visualized by chilling the gel (20), were cut out, and protein was recovered by electroelution or pulverization (22). The PAGE gel slurry was taken up in tris-glycine buffer (*p*H 8.3) and 0.1 percent SDS containing 0.05*M* β-mercaptoethanol and heated to 100°C for 5 minutes to enhance solubilization of protein.

This product was used in competition assays against ^{125}I virus for VP_3-specific antibodies and to vaccinate livestock. Figure 4 shows VP_3 antigenic reactivity by autoradiography of the LE′-VP_3 fusion protein after SDS-PAGE separation of *E. coli* proteins. Only the fusion protein LE′-VP_3 (lanes 3a and 4a) and VP_3 from FMDV (lane 5a) show reactivity with ^{125}I-labeled antibodies specific for VP_3. None of the normal *E. coli* proteins or other plasmid encoded proteins were reactive.

The purified fusion protein, LE′-VP_3, from pFM_1 and the purified virion protein VP_3 were compared by radioimmunoassay for their ability to compete with ^{125}I-labeled 140*S* virions for specific antibody to VP_3. This reaction involves

the binding of only those antibodies specific for VP_3 which recognize native virion antigenic determinants. The proteins were taken up individually in 6*M* urea, 0.05*M* β-mercaptoethanol, and 0.014*M* tris-HCl, *p*H 8.6, and quantified by analytical PAGE scanning (*20*). For the assay, 0.4 ml of guinea pig antiserum to VP_3 (1:40,000 dilution) in an isotonic borate-buffered saline solution and 0.01 ml of VP_3 protein (0.2 to 30 pmole) were incubated for 1 hour at room temperature; 0.1 ml of a solution containing ^{125}I-labeled 140*S* virions (about 20 to 30 ng) was added; and after 1 hour the antibody-antigen complex was precipitated (*23*). After centrifugation, the supernatant was removed, and radioactivity in the pellet was determined. These data (Fig. 5) show that the fusion protein (LE′-VP_3) and the VP_3 protein have similar antigenic reactivity in vitro when competing for antibody reactive with VP_3 native virion antigenic determinants.

The LE′-VP_3 fusion protein and VP_3 protein (isolated from the virion) were tested and compared for use as a polypeptide vaccine against FMD in an immune response and challenge assay. The vaccines were prepared in an oil adjuvant. Samples of the vaccine were tested and found not to contain live virus (*24*). One dose of test vaccine consisted of 3.5 ml of gel slurry material containing 250 μg of LE′-VP_3 or 140 μg of VP_3 combined with 3.5 ml of incomplete Freund-type oil (Marcol 52 oil, 10 percent Arlacel A, and 1 percent Emulugin 05). The vaccine was administered subcutaneously on days 1 and 24 for swine and on days 1 and 28 for cattle. Serum samples from six cattle and two swine inoculated with the LE′-VP_3 vaccine and two cattle inoculated with VP_3 vaccine were collected on days 7, 14, 21, 28, 35, and the challenge day as well as 14 days after challenge. The titers of the FMDV-neutralizing antibodies in these serums were determined by suckling mice protection assay (*25*) (Table 1).

The immunity elicited by the vaccines was challenged (*26, 27*) on day 42 for the cattle and on day 32 for the swine. Each group was exposed to two nonvaccinated animals of the same species, one of which was inoculated into the epithelium of the tongue with 10,000 mouse LD_{50} units of the virulent homologous strain of FMDV. A high level of protection was obtained with the LE′-VP_3 fusion protein vaccine (Table 1). The eight animals vaccinated with LE′-VP_3 did not experience generalized FMD. Of two cattle vaccinated with VP_3, isolated from the natural virus (positive control), one was fully protected, while the other had a low antibody titer and was not protected. The nonvaccinated cattle and swine inoculated with the infectious virus and contact controls developed FMD. A serum sample from each animal was taken 14 days after challenge and examined for neutralization titer and VIA (virus infection associated antigen) antibody (*28*). The absence or presence of VIA anti-

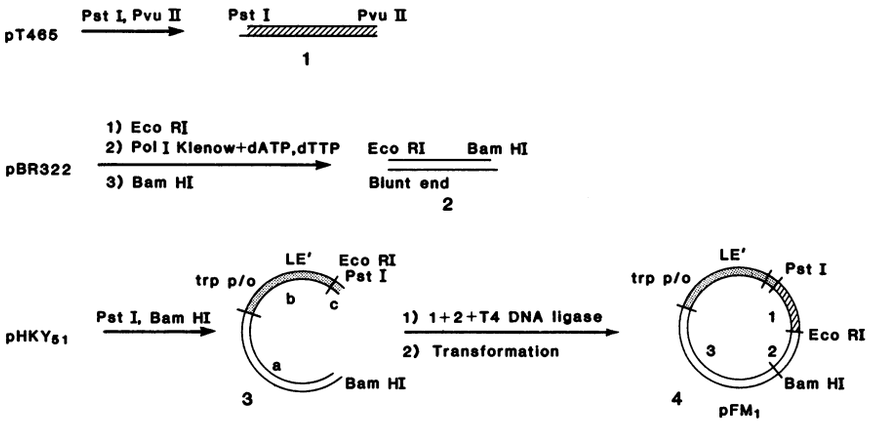
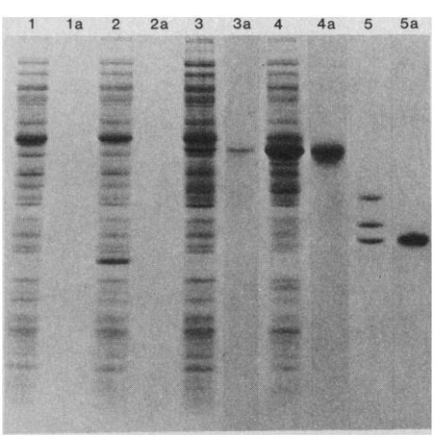


Fig. 3. Construction of the expressed vector pFM_1. (i) The cDNA insert in plasmid pT465 (Fig. 1) was cleaved with Pst I and Pvu II to give a 610-bp DNA fragment, 1, containing codons 8 to 211 of the VP_3 gene that was isolated by polyacrylamide gel electrophoresis (PAGE) and electroelution. (ii) The plasmid pBR322 (*33*) was cleaved with Eco RI and the resulting linear DNA was treated with DNA polymerase I Klenow fragment (*34*) in the presence of deoxyadenosine triphosphate (dATP) and deoxythymidine triphosphate (dTTP) to create blunt ends containing the Eco RI recognition sequence. This linear DNA was cleaved with Bam HI, and the resulting 375-bp fragment, 2, containing the promoter and the beginning of the tetracycline resistance gene was recovered by PAGE. (iii) The vector, $pHKY_{51}$, was cleaved with Pst I and Bam HI and the large fragment, *3*, recovered by PAGE. The vector, 3, is made up of three fragments. Fragment 3a is the plasmid pBR322 extending from the Bam HI site at 375 bp to the Pst I site at 3611 bp. (This Pst I site was removed with polymerase I Klenow fragment under standard conditions.) Fragment 3b consists of a DNA fragment that codes for the trp promoter-operator and 190 codons ultimately derived from the trp operon containing the attenuator deletion trpΔLE1413 (*14*). An Eco RI site has been introduced into the trp LE gene at the trp E termination codon (*15*). Fragment 3c is a small DNA fragment bounded by Eco RI and Pst I sites. This linker has the following sequence: AATTCCACTGCCTTCCACCAAGCTCTGCA. Fragment 3c joins the genes trp LE and VP_3 at Eco RI and Pst I ends into their correct reading frames (*35, 36*). The three fragments were combined and treated with T4 DNA ligase. Fragments 1 and 3 join to recreate the Pst I site, fragments 2 and 3 join to recreate the Bam HI site, and fragments 1 and 2 join, by blunt-end ligation, to create a new Eco RI site from the Pvu II site and the filled-in Eco RI site.



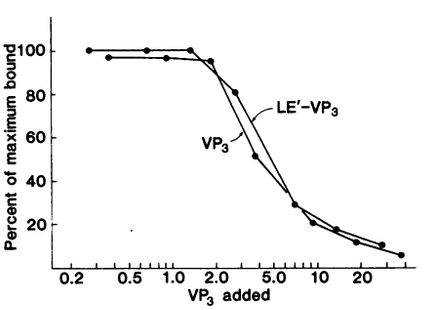


Fig. 4 (left). Polyacrylamide gel analysis of the LE′-VP_3 fusion protein. Each sample is shown in pairs. The first lane of the pair shows the total bacterial or viral protein stained with Coomassie brilliant blue. The second lane of the pair is an autoradiogram of protein that has been transferred to CNBr-treated paper and exposed to ^{125}I-labeled antibody to VP_3 (*37–39*). Lanes 1 and 1a, *E. coli* 294; lanes 2 and 2a, *E. coli* 294/pLE control in which only the LE protein was expressed; lanes 3 and 3a, *E. coli* 294/pFM_1 partially induced for fusion protein expression (tryptophan in the growth media was not completely depleted); lanes 4 and 4a, *E. coli* 294/pFM_1 fully induced; lanes 5 and 5a, FMDV A_{12} 119ab VP_1 and VP_2, VP_3 isolated from FMDV (VP_4 is not visible). Fig. 5 (right). The fusion protein LE′-VP_3 was compared to purified virion VP_3 for its ability to compete with ^{125}I-labeled 140*S* virions for VP_3-specific antibody. The 140*S* virions and VP_3 antibody were incubated in the presence of increasing amounts of added antigen (0.2 to 30 picomoles). The antigen-antibody complexes were precipitated with *Staphylococcus aureus* cells (*23*), and the pellet was measured for radioactivity.

body and the change in virus neutralization titer confirmed the results of the challenge test. Animal No. 5 vaccinated with LE′-VP_3, which developed a single foot lesion, did not develop VIA antibody nor did it show a rise in neutralization titer, an indication that systemic infection had not occurred. In contrast to the high level of response elicited by the fusion protein, one of the cattle vaccinated with VP_3 responded with a virus neutralizing antibody titer (1.6 log) that was significantly lower than those of the other vaccinated cattle and it was not immune to challenge. The cause of this single poor response is obscure, because in a previous report (*9*) six cattle responded to VP_3 contained in gel slurries with 2.7 log of neutralizing antibody and immunity to challenge.

We believe that this is the first report of an effective protein vaccine derived from the microbial expression of recombinant DNA. *Escherichia coli* transformed with plasmid pFM_1 synthesized the LE′-VP_3 fusion protein as the principal product after induction in tryptophan-depleted media. This product constituted about 17 percent of the protein in the bacterial lysate (1×10^6 to 2×10^6 molecules per bacterium). Moreover, the fusion protein appears to be stable in the cell extracts (Fig. 4). This stability to degradation may be due, in part, to the insolubility of VP_3 and its formation of aggregates (*5*). Like VP_3 isolated from virions (*8, 9*), the immunogenic activity of the purified fusion protein was stable to heating at 100°C.

It is apparent that the LE′ polypeptide domain in the LE′-VP_3 fusion protein did not mask the immunogenic activity of the VP_3 domain. The levels before challenge of neutralizing antibody elicited by the fusion protein were similar to those obtained with whole virus and VP_3 vaccines and were indicative of immunity. The levels were markedly higher for cattle (1.2 to 2.7 log) than for swine (1.3 to 1.5 log), in accord with previous reports for oil-adjuvanted whole-virus vaccine (*27, 28*).

A putative immunogenic site or collection of sites resides between methionine residues 54 and 179 (Fig. 2) because an immunogenic fragment with a molecular size of 13 kd (about 13.48 kd from amino acid composition) is derived from VP_3 by cleavage with CNBr (*4, 9*). Within this fragment, 125 amino acids in length, from the central region of VP_3, there are 36 differences in amino acid residues, including a loss of one amino acid between virus type A_{12}119ab (Fig. 2) and the 126 amino acid region reported for type O_{1K} virus (*18*). Sixteen of these differences are clustered between residues 133 and 153.

The amino acid sequences for capsid proteins VP_1 and VP_3 agree well with the sequences in Fig. 2. Thus (i) residues at positions −1 and −2, -Ser-Gln, are the reported COOH-terminus of VP_1 (*5*); (ii) residues 1 to 15 correspond to the known NH_2-terminal region of VP_3 (*9*); and (iii) residues 58 to 68 correspond to those determined near the NH_2-terminus of the immunogenically active 13-kd fragment (*9*). The -Gln-Leu at codons 211 and 212 may be the COOH-terminus for type A_{12}119ab VP_3; this locus is reported to be -Gln-Ala-Leu for a different A_{12}119 virus (*5*) and -Gln-Thr-Leu for type O_{1K} virus (*18*). These differences could be accounted for if the resulting VP_3 gene of the A_{12}119ab virus has a deleted penultimate codon as compared with both of the above viruses.

In order to elicit the protective immunogenic response demonstrated in our study, the biosynthetic LE′-VP_3 antigen must present an immunogenic site as it appears in the intact virion. The immunogenic site must survive long enough to be recognized by the immune system, and the antibodies induced must bind to virions with sufficient affinity and specificity to provide protection. There is some evidence that VP_3 elicits antibodies to several different antigenic sites, only some of which are present in the intact virion (*29*). Nevertheless, our results demonstrate that at least one site of high structural integrity and immunogenic potency which is present on the virion and on VP_3 is also present on the cloned viral polypeptide LE′-VP_3.

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35. An alternative construction contains a synthetic DNA fragment in place of fragment 3c. This fragment contains eight codons bounded by Eco RI and Pst I sites. It was produced by ligation of five synthetic oligonucleotides (AATTCATGAC, TACTGCTACTGG, TGAATCTGCA, GATTCACCAGTA, GCAGTAGTCATG) (A, adenine; T, thymine; C, cytosine; G, guanine) synthesized by the methods of R. Crea *et al.* (*36*) and cloned by the methods of D. V. Goeddel *et al.* (*16*). This fragment codes for an initial methionine amino acid followed by seven codons for the first seven amino acids of the VP_3 gene. It was used in place of fragment 3c to construct plasmid pFM_2 (data not shown).
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38. The polyacrylamide slab gel (1.27 mm by 9 cm by 14 cm) of 12.5 percent acrylamide (*39*) containing 8*M* urea (*20, 21*) was washed for 30 minutes in two changes of phosphate-buffered saline (PBS) solution. The gel was placed on CNBr-activated filter paper (*40*) for 1 hour at room temperature, and the gel was later stained with Coomassie brilliant blue. The paper was treated in PBS containing 0.1*M* glycine and 0.2 percent bovine serum albumin (BSA) at 37°C for 4 hours (or 18 hours at 4°C), washed in PBS for 10 minutes, placed in a plastic bag, and treated for 1 hour at 37°C with 5 percent guinea pig serum in PBS (GPS-PBS) followed by (7.5×10^6 to 10×10^6 cpm) ^{125}I-labeled guinea pig immunoglobulin G (IgG) specific for VP_3 in 10 ml of GPS-PBS. The VP_3-specific antibody was iodinated [100 μg of IgG, 10 μg of Iodogen (Pierce), and 500 μCi of ^{125}I (NaI) for 15 minutes at room temperature] and separated by G-25 Sephadex chromatography. After 4 hours at room temperature, the filter paper was washed with 30 ml of GPS-PBS for 1 hour, rinsed twice with 200 ml of PBS for 18 hours, blotted, air-dried, and exposed to x-ray film with the use of an intensifying screen at −60°C.
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41. We thank Karl Axelson, James Dean, Nicholas Shuot, Marla Koster, and JoAnn Henry of the Plum Island Animal Disease Center (PIADC) and Greg Weddell and Patricia Tanttila of Genentech for technical assistance. The work was greatly facilitated by the constant attendance and help of K.A. The dideoxy- sequencing was done by Richard Najarian. The oligonucleotides used in this project were synthesized by Mark Vasser, Parkash Jhurani, and Dr. Roberto Crea of Genentech. We thank Dr. J. S. Walker of PIADC's Biosafety Committee, Dr. G. Still of the USDA Science and Education Administration, Dr. H. Kryder of the USDA Vectors Committee, Dr. S. Barban of NIH-ORDA, and the NIH-RAC and FMD subcommittee for assistance, and Dr. J. J. Callis, director of PIADC, for his help and for his encouragement throughout this project.

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Regulation of Leucine Metabolism in Man: A Stable Isotope Study

Abstract. *Leucine catabolism is regulated by either of the first two degradative steps: (reversible) transamination to the keto acid or subsequent decarboxylation. A method is described to measure rates of leucine transamination, reamination, and keto acid oxidation. The method is applied directly to humans by infusing the nonradioactive tracer,* L-[^{15}*N,1-*^{13}*C]leucine. Leucine transamination was found to be operating several times faster than the keto acid decarboxylation and to be of equal magnitude in adult human males under two different dietary conditions, postabsorptive and fed. These results indicate that decarboxylation, not transamination, is the rate-limiting step in normal human leucine metabolism.*

The branched-chain amino acids leucine, valine, and isoleucine play a prominent role in the amino acid metabolism of muscle. In contrast to the other essential amino acids that are oxidized by the liver, the branched-chain amino acids are catabolized primarily by peripheral tissues such as muscle (*1*). This catabolism is thought to represent an important muscle energy source during early starvation and an important nitrogen source for muscle alanine and glutamine production for subsequent hepatic gluconeogenesis (*1*). Because of the unusual properties of the branched-chain amino acids, much research has focused on their metabolism, particularly the metabolism of leucine.

Control of leucine oxidation occurs at either or both of the first two steps of catabolism: transamination of the leucine to α-ketoisocaproate (KIC) or decarboxylation of KIC (Fig. 1). In muscle the latter step is thought to be rate controlling, although transamination activity also changes in response to various perturbations (*1*). Our knowledge of the control of leucine oxidation comes largely from studies with [^{14}C]leucine and perfused or isolated animal tissue systems. These systems can only approximate the physiology of the intact animal where multiple additional substrate and hormonal regulatory interactions are present. Moreover, species differences in leucine metabolism limit extrapolation of animal data to man.

Because the first step in leucine catabolism, transamination to KIC, is reversible (*2*), the use of a [^{14}C]leucine tracer alone supplies information on the generation of [^{14}C]KIC, but provides no insight into the rate of reamination of KIC to leucine. In this report we describe a method for measuring the rates of deamination, reamination, and oxidation of leucine in man using the safe, nonradioactive tracer L-[^{15}N,1-^{13}C]leucine.

During a primed, continuous infusion of L-[^{15}N,1-^{13}C]leucine, dilution of the dilabeled [^{15}N,1-^{13}C]leucine in plasma reflects the appearance of unlabeled leucine derived primarily from leucine release from protein breakdown and from dietary leucine intake. [^{15}N,1-^{13}C]Leucine is removed from the system by routes such as leucine incorporation into protein (protein synthesis) and leucine oxidation—the latter causes the differential release of the labels. Initial removal of ^{15}N by leucine transamination in the forward direction produces a [1-^{13}C]KIC (Fig. 1). The labeled keto acid has two fates: (i) decarboxylation to $^{13}CO_2$ or (ii) reamination to leucine, which produces singly labeled [1-^{13}C]leucine (*3*). Thus, mass spectrometric determination of the various labeled species present, [^{15}N,1-^{13}C]leucine, [1-^{13}C]leucine, and $^{13}CO_2$, allows solution of the overall model for leucine catabolism (Fig. 1).

The specific solution requires consideration of leucine carbon and nitrogen disposition. Figure 2 presents a model for leucine C oxidation (*4*). Leucine enters the free leucine pool by dietary intake (*I*) and leucine release from protein breakdown (*B*). Net leucine removal occurs by leucine incorporation into protein (*S*) and leucine oxidation (*C*). At steady state, leucine appearance will equal leucine disappearance which is net leucine C flux (Q_C):

$$Q_C = I + B = S + C \quad (1)$$

The leucine carboxyl-^{13}C label is not lost by leucine transamination to and from KIC, only by the subsequent decar-

Table 1. The effects of feeding and fasting on leucine metabolism measured with a primed, continuous infusion of [^{15}N,1-^{13}C]leucine. Data are given as means ± standard error for three subjects infused in the postabsorptive state and four subjects infused in the fed state.

Parameter	Value (μmole/kg-hour)	
	Postabsorptive	Fed
Net leucine C flux (Q_C)	83.9 ± 6.6	153.0 ± 7.5*
Leucine N flux (Q_N)	198.9 ± 23.2	273.4 ± 11.8†
Leucine oxidation (C)	10.6 ± 1.6	31.6 ± 3.2*
Leucine transamination to KIC (X_O)	121.8 ± 19.2	152.1 ± 10.9
KIC transamination to leucine (X_N)	111.2 ± 18.0	120.5 ± 8.6

*Difference between postabsorptive and fed states, $P < .005$. †$P < .05$.

Table 2. Relative values of leucine metabolism determined from data in Table 1.

Parameter	Postabsorptive	Fed
Percentage of leucine flux oxidized (C/Q_C)	11.9 ± 1.3	21.0 ± 2.5*
Percentage of KIC reaminated to leucine (X_N/X_O)	91.2 ± 0.8	79.2 ± 1.4†
Percentage of KIC oxidized (C/X_O)	8.8 ± 0.8	20.8 ± 1.4†

*Difference between postabsorptive and fed states, $P < .05$. †$P < .005$.

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