Cloned Gene of *Rickettsia rickettsii* Surface Antigen: Candidate Vaccine for Rocky Mountain Spotted Fever

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Two major protective antigens of *Rickettsia rickettsii* have been previously described. In this study, we cloned the gene encoding one of these antigens into *Escherichia coli* and tested the effectiveness of the recombinant-made product as a vaccine for Rocky Mountain spotted fever. A clone bank of R strain *R. rickettsii* DNA was made in *E. coli* K-12 by using the plasmid vector pBR322. Transformants were screened for their ability to make rickettsial antigens by reactivity with rabbit antibodies to *R. rickettsii*. One of the transformants, EM24(pGAM21), made a product reactive with two monoclonal antibodies that recognize a 155-kilodalton protein of *R. rickettsii*. One of the monoclonal antibodies was a member of a class of antibodies that react to heat-sensitive epitopes and protect mice injected with a potentially lethal dose of viable *R. rickettsii*. The cloned product contained this protective heat-sensitive epitope. In order to obtain enhanced expression, the gene was subcloned downstream of the lactose promoter on the plasmid vector pUC8. A sonic lysate of *E. coli* harboring the pUC8 subclone was used successfully as a vaccine to protect mice injected with a lethal dose of the viable *R. rickettsii*.

ROCKY MOUNTAIN SPOTTED FEVER (RMSF) is caused by a Gram-negative obligate intracellular bacterium (*Rickettsia rickettsii*). Humans are usually infected through a bite from an infected tick. Rickettsiae spread through the blood system and invade vascular endothelial cells as well as vascular smooth muscle cells of the kidneys, heart, skin, brain, and subcutaneous tissues (*1, 2*). The invaded cells are destroyed (*1, 2*) and there is a resulting increase in vascular permeability, a decrease in blood volume, and edema (*1*). Death can result from toxemia, vasomotor weakness, shock, renal failure (*3*), or respiratory or cardiac arrest (*4*).

If detected early, RMSF can be effectively treated with chloramphenicol or tetracycline; in 1983, the case fatality rate of people who received no antibiotic treatment was 7%, whereas 4% of those treated with antibiotics died (*5*). There is no satisfactory diagnostic test for early detection of RMSF, and early treatment is critical in combating the disease. Physicians must make assessments based on clinical criteria such as fever, rash, headache, and nausea. Such assessments are not always accurate (*6*) because RMSF can mimic measles, meningococcal disease, pneumonia, infectious mononucleosis, viral infections, salmonelloses, and other infections (*4*).

No effective RMSF vaccine has been made, although several whole-cell vaccines have been tried in humans. Two surface protein antigens of *R. rickettsii* have been tentatively identified as major protective antigens and possible candidates for use in subunit vaccines (*7, 8*). These proteins have apparent molecular weights of 120,000 (120K) and 155,000 (155K) (*8*). Sera from convalescent human patients (*7*) detect these proteins in radioimmunoprecipitations of iodinated surface antigens; also, monospecific polyclonal rabbit serum to the 155K antigen (antigen 2) protects guinea pigs from RMSF (*9*).

Anacker *et al.* (*7*) reported that monoclonal antibodies to the 120K and 155K antigens protected mice (protective monoclonal antibodies) against a lethal injection of viable *R. rickettsii*. The 133K and 170K antigens referred to in a previous study (*7*) are the same as the 120K and 155K antigens, respectively; Anacker *et al.* (*8*) changed the reported molecular sizes for the antigens after subjecting them to electrophoresis on 10% SDS–polyacrylamide gel electrophoresis (PAGE) gels as opposed to 12.5% gels. Anacker *et al.* (*7*) showed that the monoclonal antibodies reacted with surface proteins, as evidenced by the staining of intact cells with protein A and colloidal gold. Protective monoclonal antibodies apparently recognize heat-sensitive (conformational) epitopes. This is suggested by the observation that the protective antibodies will not recognize antigen by protein immunoblot if the rickettsiae are solubilized at 100°C before electrophoresis, but they will react if the rickettsiae are solubilized at room temperature (*8*). Recently, we found that monoclonal antibodies to the 155K protein that do recognize heat-solubilized antigen, by protein immunoblot, do not protect mice (are nonprotective) against lethal challenge. Thus, conformational integrity of the 155K antigen appears to be important in its protective ability; Dasch (*10*) reported a similar observation with monoclonal antibodies to surface protein antigens of rickettsiae of the typhus group.

The 155K and 120K proteins might be useful as immunogens for humans, but it is difficult to purify them from *R. rickettsii* because this organism grows poorly in host cells and is difficult to separate from host components. The production of the protective antigens in *Escherichia coli* would enhance antigen yields and facilitate purification. We report here the identification of an *E. coli* recombinant expressing the 155K protein. The recombinant-made product elicits protection in mice against lethal challenge with *R. rickettsii*.

DNA, purified (*11*) from the R strain of *R. rickettsii,* was partially digested with the restriction enzyme Sau 3A. The fragments were ligated into Bam HI–digested, dephosphorylated pBR322 (*12*). The ligation mixture was transformed (*13*) into *E. coli* strain EM24. Transformants making rickettsial antigens were identified by using a colony-immunoblot procedure (*14*). A recombinant was identified that reacted with serum from a hyperimmune rabbit that had been injected with intact *R. rickettsii* (*9*) and with a monospecific polyclonal antiserum to the 155K antigen (*9*). This recombinant was designated EM24(pGAM21). A restriction enzyme map of the plasmid (pGAM21) harbored by the recombinant is shown in Fig. 1.

Western blot analysis (Fig. 2) confirmed the production of the 155K protein by EM24(pGAM21). The proteins in Fig. 2 (lanes 1 to 3) were reacted with a nonpro-

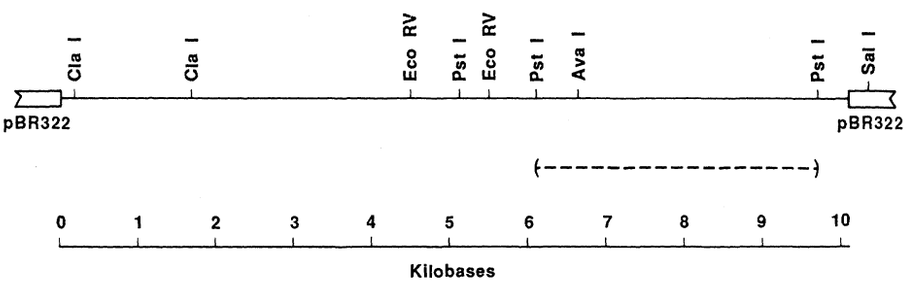

Fig. 1. Restriction enzyme map of the 10.1-kb insert in pGAM21. Dashed line denotes a 3.7-kb Pst I fragment, which was subcloned into plasmid vectors pBR322 and pUC8; its significance is described later in the text.

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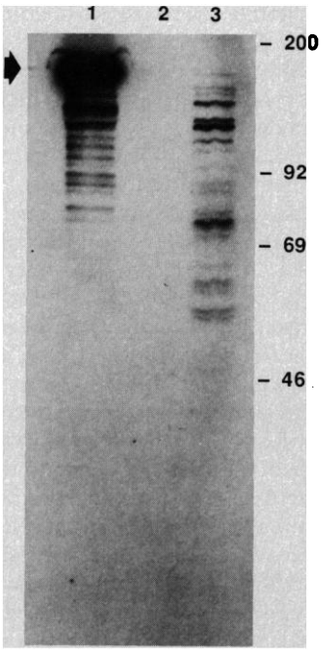

Fig. 2. Protein immunoblot analysis of the cloned protein. Whole cells were solubilized in 1× sample buffer (*21*) at 100°C for 7 minutes. The lysates were subjected to SDS–polyacrylamide gel electrophoresis (*21*) and then protein immunoblotting (*22*). Lane 1 contains 5 μg of *R. rickettsii* protein; lane 2, 40 μg (protein) of *E. coli* strain EM24 harboring pBR322; and lane 3, 40 μg (protein) of the recombinant EM24(pGAM21). The arrow indicates the 155K protein, and the numbers (×1000) on the right indicate molecular weights.

tective monoclonal antibody to the 155K protein. EM24(pGAM21) produced several peptides that reacted with this antibody. The lower molecular weight bands might have been due to partial proteolytic degradation of the cloned product. It is not uncommon for exogenous proteins to undergo rapid degradation in *E. coli* (*15*). The monoclonal antibody also reacted with multiple bands of the rickettsial proteins (Fig. 2, lane 1). These lower molecular weight bands may also have been degradation or breakdown products of the 155K protein. The multiple bands seen with the recombinant may reflect the susceptibility of the protein to proteolysis or breakdown.

We used Tn5 mutagenesis (*16*) to localize the gene encoding the 155K protein to a 3.7-kb Pst I fragment (Fig. 1). In theory, a 3.7-kb fragment of DNA has the capacity to encode a protein with a maximum size of only 137K, and it would require a gene of 4.1 kb in length to encode a 155K protein (*17*). The 155K molecular weight assigned to this protein is only an apparent molecular weight based on relative migration in SDS-polyacrylamide gels, and its true molecular weight is not known. It is possible, however, that only a portion of the gene was present on the 3.7-kb Pst I fragment.

The 3.7-kb Pst I fragment was subcloned into pBR322 to yield pGAM4 and into pUC8 (*18*) to yield pGAM22. Dot blot analysis (Fig. 3) revealed that the protein encoded by pGAM4 was recognized by both a nonprotective and a protective monoclonal antibody. More important was the finding that the epitope on the cloned protein, which is recognized by the protective monoclonal antibody, is heat-sensitive.

Since the cloned product was reactive with a protective monoclonal antibody, we wished to determine whether it would immunize mice against a lethal challenge with viable rickettsiae. Sonic lysates of *E. coli* strain JM107 harboring pUC8 and strain JM107 harboring pGAM22 (pUC8 with the gene encoding the 155K antigen inserted at the Pst I site) were used to vaccinate mice. The mice were challenged after 1 week. The results are shown in Table 1. We also performed passive protection experiments [as described in (*8*)] with the protective and nonprotective monoclonal antibodies used in this study. Each of ten mice was inoculated with a mixture of the nonprotective antibody 13-1D4D9 (see Fig. 3) and a 2 LD_{50} (median lethal dose) of viable *R. rickettsii*, and none of them survived; another nine mice were inoculated with a mixture of the protective antibody, 7-9D6D8, and a 2 LD_{50} of viable *R. rickettsii*, and all of them survived.

Thus, we have shown that the gene for a major surface antigen of *R. rickettsii* can be cloned and expressed in *E. coli* and that the product of this cloned gene retains its protective activity in mice. All vaccines used previously to protect mice from RMSF upon challenge with viable *R. rickettsii* have also been effective in guinea pigs (*19, 20*). This suggests that the subunit vaccine we have described may also be effective in other species. The production of cloned vaccines would indeed be valuable in the control of RMSF.

Table 1. Protection of mice against lethal injection of *R. rickettsii*. Sonic lysates of the *E. coli* strain JM107 harboring pUC8 and JM107 harboring pGAM22 were prepared as described in Fig. 3. Male Swiss mice (3 weeks old) of the Rocky Mountain Laboratories were vaccinated by intraperitoneal injection with the indicated amount of the sonic lysates. The mice were challenged with (2 LD_{50}) intravenous injection of viable R strain *R. rickettsii* 1 week after vaccination. Survivors were counted 24 hours after challenge.

Strain	Dose (microgram of protein	Number of mice	Survivors
JM107 (pUC8)	235	4	0
	470	4	0
JM107 (pGAM22)	112	4	1
	235	4	4
	470	4	3

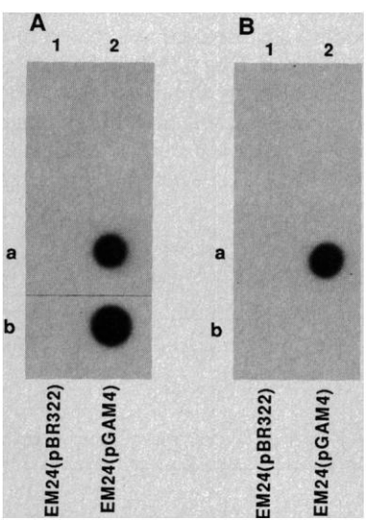

Fig. 3. Dot blot (Bio-Rad) analysis demonstrating the presence of a heat-sensitive (protective) epitope on the cloned product. *Escherichia coli* strain EM24 harboring pBR322 and strain EM24 harboring pGAM4 were grown to midlogarithmic phase and washed three times in 0.067*M* phosphate-buffered saline. The cells were sonicated, and unbroken cells were removed by centrifugation. The samples in (**A**) were reacted with a nonprotective monoclonal antibody (13-1D4D9). At location 1a is the result of analysis of 28 μg (protein) of the sonic lysate from EM24(pBR322) held at room temperature prior to loading; at location 1b is the result of analysis of 28 μg of the lysate heated in a boiling water bath for 7 minutes before loading. At location 2a is the result of analysis of a 28-μg sample of EM24(pGAM4) sonic lysate kept at room temperature, and at location 2b is the result of analysis of a 28-μg sample of the heated sonic lysate. The loading pattern in (**B**) was the same as in (A), but (B) was reacted with a protective monoclonal antibody (7-9D6D8). The 7-9D6D8 was produced and characterized as described (*7*); production and characterization of 13-1D4D9 were the same as for 7-9D6D8 except that the rickettsiae were heated at 56°C for 15 minutes before injection into mice.

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Unique Forms of the *abl* Tyrosine Kinase Distinguish Ph¹-Positive CML from Ph¹-Positive ALL

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In the Philadelphia chromosome (Ph¹) of chronic myelogenous leukemia (CML), the c-*abl* gene on chromosome 9 is translocated to *bcr* on chromosome 22. This results in the expression of a chimeric *bcr-abl* message that encodes the P210$^{bcr\text{-}abl}$ tyrosine kinase. The cells of 10% of acute lymphocytic leukemia patients (ALL) carry a cytogenetically similar Ph¹ translocation. We report that Ph¹-positive ALL cells express unique *abl*-derived tyrosine kinases of 185 and 180 kilodaltons that are distinct from the *bcr-abl*–derived P210 protein of CML. The appearance of the 185/180-kilodalton proteins correlates with the expression of a novel 6.5-kilobase messenger RNA. Thus, similar genetic translocations in two different leukemias result in the expression of distinct c-*abl*–derived products.

THE LEUKEMIC CELLS OF MORE than 90% of chronic myelogenous leukemia (CML) patients carry the t(9;22) (q34;q11) that generates the Philadelphia chromosome (Ph¹) (*1*). One molecular consequence of Ph¹ is the translocation of the c-*abl* oncogene on chromosome 9 into a 5.8-kb breakpoint cluster region in the *bcr* gene (*2*). Although the breakpoints on both chromosomes are variable, RNA-splicing generates a unique 8-kb *bcr-abl* chimeric message that is larger than the normal 6- and 7-kb c-*abl* transcripts seen in most tissues (*3, 4*). This 8-kb message encodes a phosphoprotein (P210$^{bcr\text{-}abl}$) that contains *bcr*-encoded NH_2-terminal sequences and a c-*abl*–derived COOH-terminal segment (*5*). The structure of P210$^{bcr\text{-}abl}$ is reminiscent of the P160$^{v\text{-}abl}$ transforming protein encoded by the *gag-abl* gene of Abelson murine leukemia virus. As a consequence of NH_2-terminal structural alteration, both P210$^{bcr\text{-}abl}$ and P160$^{v\text{-}abl}$ have similar tyrosine kinase activities that are distinct from the activity of the normal c-*abl* product, P145$^{c\text{-}abl}$ (*6*). In the case of P160$^{v\text{-}abl}$, the tyrosine-specific kinase activity is essential for cellular transformation (*7*). By analogy, P210$^{bcr\text{-}abl}$ tyrosine kinase activity may play an important part in the pathogenesis of CML.

P210$^{bcr\text{-}abl}$ expression may be considered diagnostic of Ph¹-positive CML since this protein is found in a wide range of Ph¹-positive CML cell lines and patient samples (*8, 9*). Ph¹ is also found in approximately 10% of patients with human acute lymphocytic leukemias (ALL) and is indistinguishable from the Ph¹ of CML by cytogenetics (*10*). In contrast to CML, the breakpoint cluster region is not involved in the translocation in at least some Ph¹-positive ALL patients (*11*). We reasoned, therefore, that if altered c-*abl* expression occurs without the usual breakpoint cluster rearrangement in Ph¹-positive ALL, the molecular mechanism of expression would be different from that in Ph¹-positive CML. This could result in the expression of a novel c-*abl* product whose structure and/or function would be characteristic of Ph¹-positive ALL and distinct from Ph¹-positive CML.

By means of site-directed rabbit antisera specific for different regions of the v-*abl* protein (*12*), we examined c-*abl* protein expression in the blood and bone marrow of several CML and ALL patients (*13*). The c-*abl* proteins were precipitated with the immune sera but not with the preimmune control antisera (Fig. 1, lanes 1 and 2). Autophosphorylation of P210$^{bcr\text{-}abl}$ and, to a lesser extent, P145$^{c\text{-}abl}$, was detected in the CML-derived K562 cell lysates (Fig. 1A) as

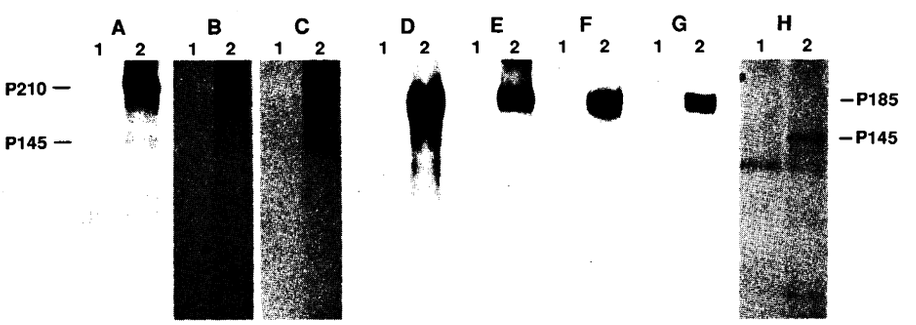

Fig. 1. In vitro kinase analysis of Ph¹-positive CML and Ph¹-positive ALL *abl* proteins. Ph¹-positive CML proerythroblastic cell line K562 (**A**); Ph¹-positive CML blast-crisis patients 146-03-37 (**B**) and 143-57-16 (**C**); Ph¹-positive ALL patients 9556 (**D**), 045-14-02 (**E**), 9254 (**F**), and 9312 (**G**); and a Ph¹-negative ALL patient 9913 (**H**). Patient samples were thawed and diluted into 15 ml of RPMI containing 10% fetal bovine serum, then incubated for 2 to 4 hours in CO_2 at 37°C. Cells were then washed and lysed in kinase lysis buffer (KLB) (*6*). Lysates were immunoprecipitated overnight with normal rabbit sera (lane 1) or with rabbit antisera anti-pEX5 specific for the COOH-terminal region of v-*abl* (lane 2) (*12*). Immune complexes were collected on protein A–Sepharose (Pharmacia) and kinase reactions were run in the presence of 20 μCi of ^{32}P-labeled adenosine triphosphate for 30 minutes at 30°C to get maximal autophosphorylation (*6*). Reactions were terminated by washing twice in KLB plus 5 m*M* EDTA. Precipitated proteins were recovered by boiling in sample buffer and diluted 20-fold in KLB according to Konopka *et al.* (*5*). Samples were reprecipitated with antisera specific for the *abl* kinase domain (anti-pEX2) (*12*), collected on protein A–Sepharose, then analyzed by 8% SDS–polyacrylamide gel electrophoresis followed by autoradiography. Approximate sizes of radiolabeled proteins were determined by comparison with nonradioactive, prestained high molecular weight standards (BRL). Exposure times were for 2 to 24 hours in panels A to G and for 14 days in panel H.

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