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Original Investigation

Antibodies to Lassa virus Z protein and nucleoprotein co-occur in human sera from Lassa fever endemic regions

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Abstract. It is not known whether the small 11-kDa Z protein of Lassa virus is immunogenic during human Lassa virus infection. To obtain evidence for the existence of an antibody response and to test the suitability of these antibodies for serosurveys, sera from Lassa fever endemic regions (Guinea and Nigeria, n=75) were tested for co-reactivity to Z protein and nucleoprotein (NP). Sera from a non-endemic region (Uganda, n=50) served as a specificity control. Z protein and NP were expressed in *Escherichia coli*, affinity-purified, and used as antigen in Western blot. Indirect immunofluorescence (IIF) with Lassa virus-infected cells was performed for comparison. Due to high unspecific reactivity of the African sera, Western blot testing was performed with a 1:1,000 serum dilution. Under these conditions, none of the control sera but 12% of the sera from endemic regions co-reacted with both Z protein and NP. Reactivity to Z protein was significantly associated with NP

reactivity ($P<10^{-6}$). NP and Z protein-specific antibodies were co-detected in 33% of the IIF-positive sera and in 5% of the IIF-negative sera ($P=0.001$). These data provide evidence for appearance of antibodies to Z protein and NP following Lassa virus infection. A recombinant blot for detection of both antibody specificities seems to be specific but less sensitive than IIF.

Keywords. Lassa virus - Antibody response - Immunofluorescence - Western blot

Introduction

Lassa virus belongs to the family *Arenaviridae* and is endemic in West African countries. Transmission of the virus from its rodent reservoir to humans causes Lassa fever which is associated with a wide spectrum of clinical manifestations including organ failure, encephalopathy and hemorrhage. Up to 20% of susceptible persons seroconvert to Lassa antibodies per year in selected villages in Sierra Leone, often without experiencing severe illness [*8*]. Serosurveys reveal a prevalence of Lassa antibodies of up to 50% in some areas of West Africa [*7, 8, 10, 13*]. Lassa virus expresses only four proteins, two of which - nucleoprotein (NP) and glycoprotein - have been shown to induce antibodies following infection [*2, 6, 7, 10*]. An NP-specific T cell response has recently been demonstrated in persons from Lassa fever endemic regions with serological evidence of previous Lassa virus infection [*11*]. Whether the newly identified 11-kDa Z protein [*3*] elicits an immune response upon Lassa virus infection is not known. There are also no corresponding data for other arenaviruses. According to studies with lymphocytic choriomeningitis virus, the Z protein is a virion component and physically interacts with NP [*12*]. This interaction may facilitate the simultaneous recognition of both proteins by the immune system. However, the small size of the Z protein and, thus, the potential lack of appropriate B and T cell epitopes may interfere with its immune recognition. In a first attempt to characterize the immune response to Z protein, we studied whether antibodies to Z protein co-occur with antibodies to Lassa virus NP in sera from Lassa fever endemic regions. The suitability for serosurveys of a recombinant blot detecting these two antibody specificities was tested by comparison with immunofluorescence using Lassa virus-infected cells.

Materials and methods

Human sera

Sera were collected during cross-sectional epidemiological studies from individuals living in Lassa fever endemic regions in West Africa (Guinea, n=60 [*9*]; Nigeria, n=15). Sera collected in a non-endemic region in Central Africa (Uganda, n=50 [*4*]) were tested as specificity control. All serum samples had been stored at -20°C.

Construction of expression plasmid

Plasmid-integrated cDNA of Lassa virus Z protein of strain Josiah (updated GenBank sequence U73035.2; kindly provided by M. Salvato, University of Wisconsin, Madison) was amplified with primers Z-5' and Z-3' (Z-5': TTCGGCTTGGATCCGGAAACAAGCAAGCCAAAGCCCCA; Z3': CGGCTTCTAAGCTTAGGGACTGTAGGGTGGGGGTC), which introduce *Bam*HI and *Hind*III sites (underlined) into the fragment for cloning. The PCR products were cleaved with *Bam*HI and *Hind*III, and inserted into vector pQE-30 (Qiagen, Hilden, Germany). Due to the cloning strategy, the N terminus of Z protein was directly fused with the histidine (His) affinity tag encoded by the vector. To prevent potential internal translation initiation, the authentic start codon of the Z protein was mutated to serine. The correct sequence of the entire His-Z protein coding region was verified by

sequencing. The cloning of a His-tagged NP of strain Josiah has been described previously [*10*].

Protein expression

His-tagged Z protein and NP were expressed in *E. coli* and purified by Ni-NTA chromatography according to the manufacturer's protocols [*1*] with minor modifications. Z protein was expressed in *E. coli* JM 109. Cells were pelleted, resuspended in 5 ml buffer A (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl) supplemented with 10 mM imidazole/1 mM PMSF, and lysed by two freeze-thaw cycles and sonication. Since a substantial fraction of the protein localized in the soluble fraction (Fig. 1A), cell debris was removed by centrifugation and the cleared cytoplasmic lysate was incubated with 1 ml Ni-NTA slurry at 4°C for 1 h. The material was loaded onto a column and washed with buffer A/50 mM imidazole. Z protein was eluted in 0.5-ml fractions of buffer A/250 mM imidazole. NP was expressed in *E. coli* BL21. Since this protein was in the insoluble fraction, it was extracted from pelleted cell debris using 8 M urea and sonication. The urea-soluble material was incubated with Ni-NTA slurry at 4°C for 1 h, loaded onto a column, and NP was eluted by pH gradient in 1 ml fractions as described [*1*]. Purified Z protein and NP were essentially free from contaminating proteins (Fig. 1). The fractions with the highest protein content were pooled and the protein concentration was determined using the Bradford assay (0.3-0.5 mg protein/ml). Aliquots of the eluates (7 µg Z protein and 3.5 µg NP per cm gel width) were separated by SDS-15% polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane (Schleicher & Schüll, Germany). The membrane was stained with Ponceau S and cut into 2- to 3-mm-wide strips. NP and Z protein bands (2 µg Z protein and 1 µg NP per strip) were located exactly 1 cm from top and bottom of the strip, respectively, allowing precise allocation of signals.

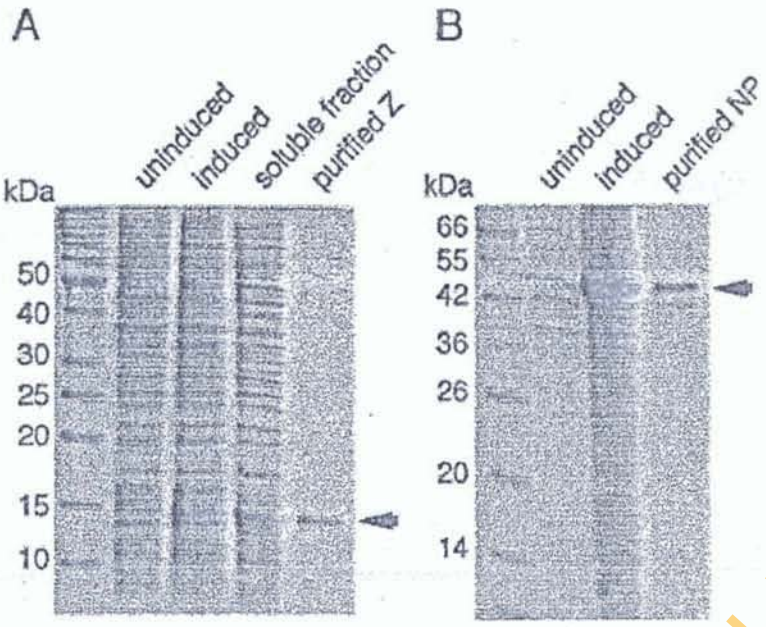

Fig. 1. Expression of Lassa virus Z protein (A) and Lassa virus NP (B) in *E. coli* and purification of the proteins by Ni-NTA chromatography. Uninduced and induced bacteria as well as the purified protein were separated in SDS-polyacrylamide gel and stained with Coomassie blue. The positions of the recombinant proteins are marked by *arrows*

Immunoblot assay

All steps were carried out at room temperature with gentle agitation. Strips were blocked with a 5% solution of non-fat dry milk for 2 h. Subsequently, they were incubated for 2 h with human serum diluted 1:1,000 in TRIS-buffered saline (TBS) containing 5% dry milk, 0.5% Tween 20, and 1% NP-40. The strips were washed three times for 15 min with TBS/0.5% Tween 20/1% NP-40 and incubated for 1 h with rabbit anti-human IgG-peroxidase conjugate (Mikrogen, Martinsried, Germany) diluted 1:5,000 in TBS/1% dry milk/0.1% Tween 20. Following two washing with TBS/0.1% Tween 20 and one with 100 mM TRIS-HCl pH 7.8/100 mM NaCl, the strip was incubated for approximately 10 min in tetramethylbenzidine (TMB; Mikrogen).

Indirect immunofluorescence

Lassa virus (strain Josiah) was propagated in Vero cells in the biosafety level 4 facility. For indirect immunofluorescence (IIF), the cells were spread onto immunofluorescence slides, air-dried, and acetone-fixed. A mouse IgG monoclonal antibody (mAb) to Lassa virus NP [5] diluted 1:30 was incubated with the cells for 15 min at 37°C. Without washing, serum in phosphate-buffered saline (PBS) was added to a final dilution of 1:20 and incubation was continued for 1 h at 37°C. Cells were washed for 15 min in PBS at room temperature. Human IgG was detected by incubation with fluorescein isothiocyanate (FITC)-conjugated anti-human IgG (Dianova) diluted 1:250 for 20 min at 37°C. Potential cross-reactivity to the mouse mAb was blocked by adding 5% normal mouse serum. After washing, the mAb was detected by incubation with anti-mouse IgG-rhodamine (Dianova) diluted 1:200 for 20 min at 37°C. Signals were detected with a Leica DMLS fluorescence microscope.

Results

African sera often display a high unspecific background reactivity in serological assays. Therefore, in pilot experiments with a test panel of African sera the amount of antigen on the blot, the dilution of serum and secondary antibody, the composition of the binding buffer, the incubation time, and the type of TMB were optimized. Stringent binding conditions with a high dilution of serum (1:1,000) and conjugate (1:5,000), and a high concentration of two detergents (0.5% Tween 20 and 1% NP-40) in the binding buffer were found to suppress unspecific reactivity. It is noteworthy that a panel of sera from German blood donors did not show unspecific reactivity with Z protein when tested at a dilution of 1:200. Although both proteins were affinity-purified to near homogeneity (Fig. 1), some sera reacted with a protein of lower electrophoretic mobility than NP (Fig. 2A). Binding to this protein was independent of the reactivity to Z protein or NP, and was therefore most likely caused by antibodies to a contaminating bacterial protein. Additional bands below that of NP appeared only in sera with strong reactivity to NP (Fig. 2A) and, therefore, probably resulted from a minor fraction of co-purified NP cleavage products. A corresponding faint band was seen in Coomassie-stained gel loaded with the NP eluate (Fig. 1B, lane purified NP). However, neither of the additional bands affected interpretation of the blot. Positive reactions with either Z protein or NP were confirmed in independent experiments with a different lot of strips, demonstrating the reproducibility of the method.

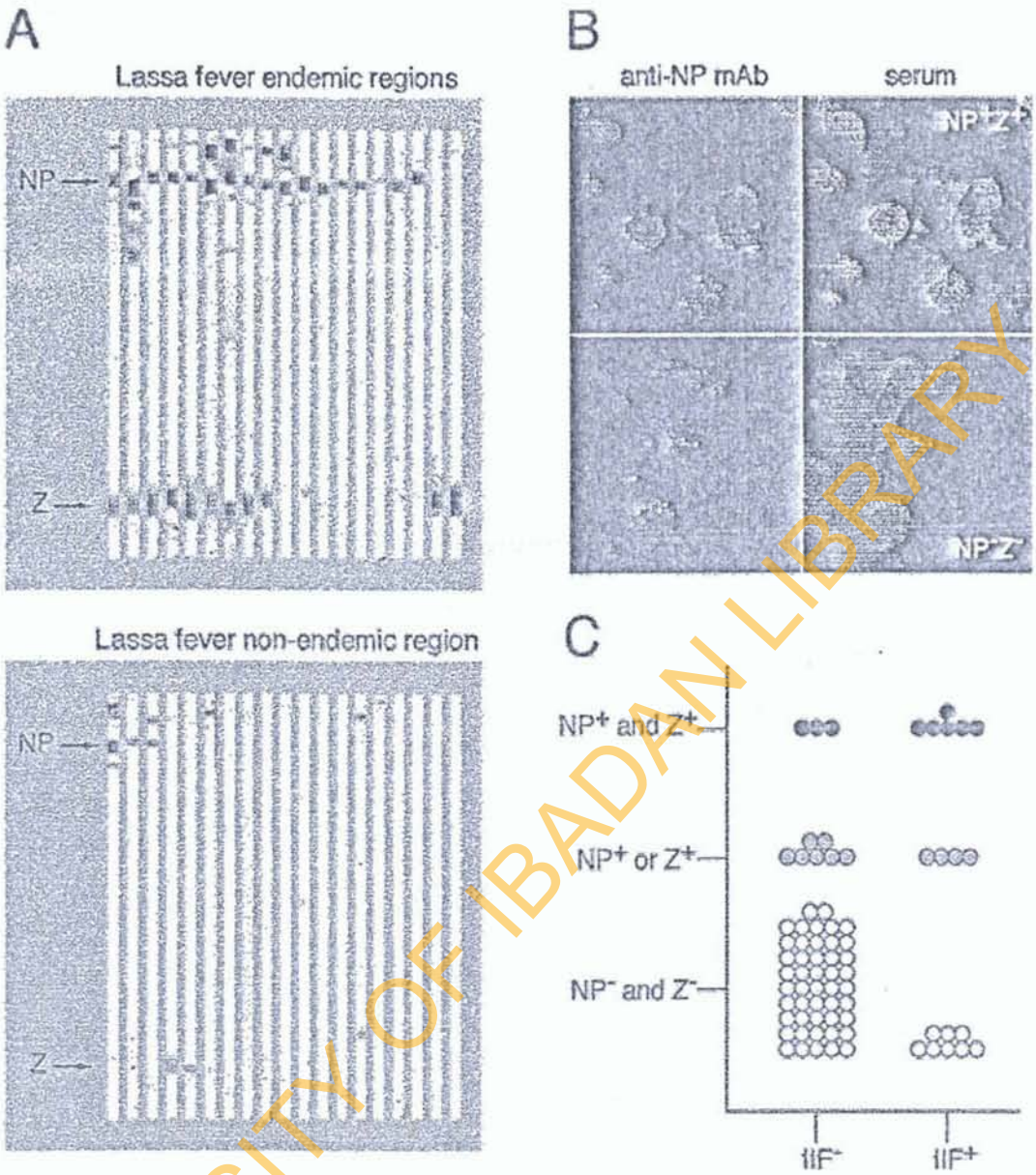

Fig. 2. A Western blot testing of human sera from Lassa fever endemic (Guinea and Nigeria) and non-endemic regions (Uganda) for antibodies to Z protein and NP. The results of representative experiments, which include all sera reacting with Z protein or NP, are shown. **B** Double-staining IIF using an NP-specific mAb (*left*, rhodamine) and serum diluted 1:20 (*right*, FITC). The results of two representative sera (*top* and *bottom*) are shown. **C** Correlation of the IIF results with the results of the recombinant blot. Each circle represents one serum sample from Guinea or Nigeria (n=75) (*NP* nucleoprotein, *IIF* indirect immunofluorescence, *mAb* monoclonal antibody)

Applying the optimized assay conditions, none of the 50 sera from Uganda, where Lassa fever is not observed, reacted with both Z protein and NP. Five sera reacted either with Z protein or with NP, but most of them weakly (Fig. 2A, bottom). In contrast, of the 75 sera collected in high endemic areas for

Lassa fever, Guinea and Nigeria, 11 (15%) reacted with Z protein and 17 (23%) reacted with NP (Fig. 2A, top). Reactivity with Z protein was found in 9 of the 17 (53%) sera reacting with NP, but in only 2 of the 58 (3.4%) sera that did not react with NP. Thus, the occurrence of antibodies to Z protein was statistically significantly associated with the presence of antibodies to NP ($P<10^{-6}$, χ^2-test).

Taken together, the results with the sera from the non-endemic region demonstrate a high specificity of the assay, in particular if reactivity to both proteins is considered. Therefore, the frequent co-reactivity of sera from Lassa fever endemic regions with Z protein and NP strongly suggests that the assay detects NP and Z protein-specific antibodies resulting from Lassa virus infection rather than unrelated cross-reactive antibodies.

To evaluate the suitability of the recombinant blot for serosurveys, it was compared with an IIF test using Lassa virus-infected cells, which is the current standard method for detecting Lassa virus-specific antibodies. The IIF test was improved by the following modification. In parallel with the human sera, cells were incubated with a Lassa virus NP-specific mAb. While the mAb was detected with a rhodamine-labeled secondary antibody to mouse IgG (Fig. 2B, left), human antibodies were detected with a FITC-labeled antibody to human IgG (Fig. 2B, right). Only if both antibodies recognized identical structures within the cells, was the test interpreted as positive (Fig. 2B, compare left and right panels). This double staining increases the specificity of the IIF test, since unspecific signals can be disregarded, as well as the sensitivity, since faint signals that may normally escape detection within a high background can be specifically searched for in infected cells (Fig. 2B, bottom right). Lassa virus-specific antibodies were detected by this IIF test in 24% of the sera from Guinea and Nigeria. One third of the IIF-positive sera was also positive for both Z protein and NP-specific antibodies, while 95% of the IIF-negative sera were negative for one or both of these antibody specificities (Fig. 2C). Although the results of both tests showed a significant correlation ($P=0.001$, χ^2-test), which corroborates the conclusion that the recombinant blot detects NP and Z-protein-specific antibodies resulting from Lassa virus infection, the blot seems to be less sensitive than IIF.

Discussion

This study shows that antibodies to Z protein and NP co-occur in sera from Lassa fever endemic regions. The recombinant blot test for detection of these antibody specificities was less sensitive than a double-staining IIF technique for detecting Lassa virus-specific antibodies.

The preparation of serological tests for Lassa virus antibodies still relies mainly on propagation of Lassa virus under biosafety level 4 conditions. Since these facilities are not available in West Africa, it is advisable to develop diagnostics which do not depend on these laboratories. This study describes the first recombinant serological assay that combines two Lassa virus proteins. Using this assay, evidence is provided that Z protein-specific antibodies appear following Lassa virus infection, though less frequently than those directed to NP. Definitive proof would require the demonstration of their appearance in consecutive sera taken during the course of Lassa virus infection. Unfortunately, such a seroconversion panel was not available. A major problem that arose during the optimization process of the blot was the high unspecific reactivity of the African sera. The background staining at the Z protein and NP positions necessitated a fivefold higher dilution of these sera compared with the German serum panel. This is probably one reason for the reduced sensitivity of the recombinant test compared with IIF and an NP dot blot assay published previously [*10*]. The double-staining IIF technique allows the identification of even very faint Lassa virus-specific signals in the face of a high background staining. Of advantage in terms of the specificity of the Western blot assay was the possibility of co-detection of antibodies to two different proteins. While (unspecific) reactivity to either Z protein or

NP was observed in some sera from the non-endemic region, none of these sera reacted with both proteins. Therefore, despite its lower sensitivity compared with IIF and NP dot blot [*10*], the recombinant NP/Z Western blot may be applicable if very high specificity rather than sensitivity is required. It is noteworthy that three sera were anti-NP and anti-Z protein positive in the recombinant blot but negative in IIF (Fig. 2C). Each of the two blots and IIF seem to detect different antibody specificities, probably depending on the different degree of denaturation of the proteins in the assays. Therefore, the blot techniques may be used in serosurveys in addition to IIF to confirm as well as to extend the IIF results. On the other hand, it is doubtful whether a Western blot technique using highly denatured proteins, allowing mainly detection of antibodies directed to linear epitopes, can approach the sensitivity of the IIF test at all. Our data may suggest that efforts in the development of sensitive recombinant assays for Lassa virus should focus on the ELISA format. The simple purification procedure of the Z protein from the cytoplasm may facilitate the application of native Z protein in such assays.

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