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Serodiagnosis of Crimean–Congo haemorrhagic fever

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SUMMARY

Several methods for demonstrating antibody to Crimean–Congo haemorrhagic fever virus were compared on serum samples taken from 101 patients during the acute stage of illness and at intervals for up to 59 months thereafter, with emphasis on early detection of the immune response. The deaths of 23 patients on days 5–14 of illness were ascribed to the effects of the disease; two patients died later from other causes. Very few of the patients who died from the acute illness mounted an antibody response detectable by the methods tested. Four patients who died and 18 who recovered were treated with immune plasma collected from recovered patients. Treated patients acquired IgG antibody from the plasma, but it was possible to discern the onset of an endogenous IgM response in those individuals who survived the disease by all of the methods tested. Indirect immunofluorescence (IF) tests detected IgM and/or IgG antibodies at the earliest on day 4 of illness in about 10% of patients who survived the disease, and by day 9 all survivors had antibodies demonstrable by IF. A biotin-streptavidin IF technique offered no advantage over the standard IF test for the early detection of IgG antibody, but demonstrated higher antibody titres and detected IgM antibody earlier in about a quarter of the patients tested. An IgM-capture enzyme-linked immunoassay (ELISA) and an IgG sandwich ELISA demonstrated higher antibody titres than did IF tests, and detected antibody responses at an earlier stage of infection than did IF tests in about one-fifth of patients, but the reverse was true in a similar proportion of instances. A competition ELISA, which detected total antibody activity, produced lower titres than did the IgM and IgG ELISAs, but yielded results which were in close agreement with the findings in IF tests. It was concluded that the IF tests were most convenient for use in making a rapid serodiagnosis of the disease.

INTRODUCTION

Crimean–Congo haemorrhagic fever (CCHF) is a tick-borne virus of Africa, Asia and eastern Europe that causes human illness with an approximately 30% fatality rate [1]. The virus has a propensity to cause nosocomial infections, and hence rapid diagnosis of the disease is important for the treatment of patients, and

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for the protection of medical and laboratory staff [2–4]. The diagnosis is generally confirmed in the laboratory by isolation of virus, demonstration of seroconversion or detection of specific IgM antibody [5]. Virus can be isolated in 1–6 days in cell cultures, but the method is less sensitive for the isolation of low concentrations of virus than the use of suckling mice which, however, takes 6–9 days [5]. Virus antigen can be detected in ticks and in tissues of infected mice and humans, by passive haemagglutination or enzyme-linked immunoassay (ELISA), but positive results were obtained in only about half of the patients from whom sera were tested during the first 2 weeks of illness, with greatest success being attained in severe and fatal infections with the most intense viraemias [4, 6–10]. Serological tests formerly used for the detection of antibody to the virus, such as complement fixation, haemagglutination-inhibition and reversed passive haemagglutination-inhibition, lacked sensitivity and reproducibility, but indirect immunofluorescence (IF) detects IgG and IgM antibody responses by days 7–9 of illness in all survivors of the infection [11–14]. Specific IgG and IgM responses can also be detected by ELISA, but tests have as yet been applied to very few patients [9, 11].

In the present study, several serological methods were compared on sera from 101 confirmed CCHF patients, with particular reference to early detection of antibody. The methods included IF tests using both fluorescein-labelled and biotinylated conjugates for the detection of IgG and IgM antibody, an IgM-capture ELISA, a sandwich ELISA for IgG antibody, and a competition ELISA (CELISA) which demonstrates total antibody activity. The results of IF tests on the sera of 50 of the patients, and of ELISA tests on sera of 5 of them, have been reported previously [11].

MATERIALS AND METHODS

Antigen and antisera

Sucrose-acetone extracted antigen was prepared from suckling mouse brain infected with South African CCHF isolate 4/81 [15] and inactivated with 0·1% beta-propriolactone [16]. Monoclonal antibody 6E5, specific for viral nucleocapsid protein, had been prepared against South African CCHF isolate 41/84 [17]. Anti-CCHF horseradish peroxidase (HRPO) conjugate was prepared from purified immune rabbit immunoglobulin [18, 19].

Serum samples

A total of 667 serum samples were collected at various intervals from the day of onset of illness up to 59 months later from 101 cases of CCHF diagnosed from February 1981–February 1992 (Fig. 1). Sera taken during the acute illness were routinely tested on receipt at the laboratory by IF for IgG and IgM antibody activity to agents associated with viral haemorrhagic fevers in Africa, inoculated into mice and cell cultures for the isolation of virus, and subjected to diagnostic tests for other aetiologic agents as appropriate, after which residual samples were stored at −70 °C for further tests. Post-convalescent sera were taken as the opportunity arose to monitor the persistence of antibody activity, or to assess the potency of immune plasma collected for therapeutic use. Adequate samples of 546 sera from 96 patients remained available for testing by ELISA for IgM antibody

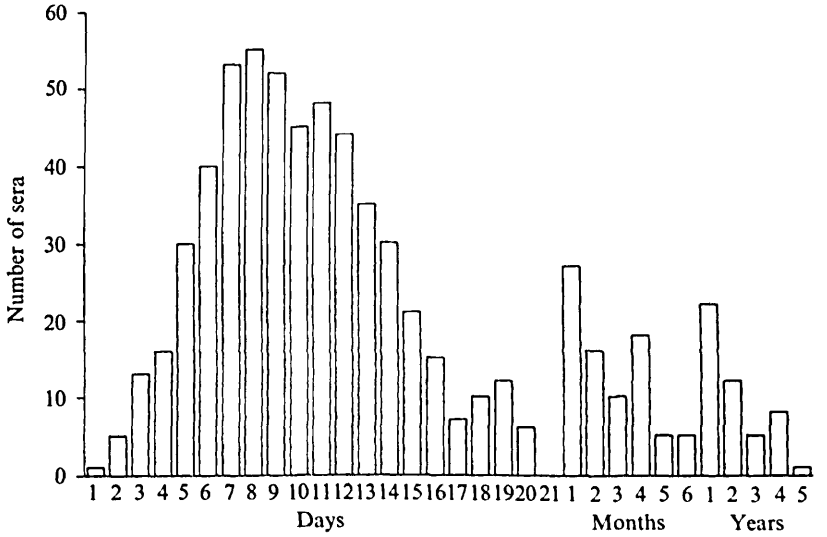

Fig. 1. Histogram showing the numbers of Crimean–Congo haemorrhagic fever patients from whom serum samples were received at the indicated intervals after onset of illness.

and by CELISA for total antibody activity to CCHF virus, whilst 425 of the sera from 86 patients were available for testing by sandwich ELISA for IgG antibody. A total of 92 sera taken from days 1–9 of illness from 39 patients were tested by the biotin-streptavidin IF method to determine if this was more sensitive than the routine IF for early detection of the immune response.

IF tests

Sera were tested by IF at doubling dilutions from 1/8 upwards for IgG and IgM antibody activity as described previously [5], using antigen slides prepared from infected cell cultures, and fluorescein-labelled anti-immunoglobulin conjugates (Cappel, Organon Teknika nv, Turnhout, Belgium). Sera with IgM antibody activity were retested at starting dilutions of 1/12 or 1/16 after treatment for removal of rheumatoid factor with commercial reagents (RF Absorbent, Behringwerke AG, Marburg, Germany, or Serum Pretreatment Reagent, Whittaker Bioproducts, Walkersville, Md, USA) used according to the manufacturers' instructions. Biotinylated anti-human IgG and IgM, and streptavidin-fluorescein conjugates (Zymed Laboratories Inc., San Francisco, Ca, USA) were used according to the manufacturer's instructions. In all of the serologic techniques, including IF tests, trials were done to establish the minimum incubation periods required to obtain reproducible results, and titres of sera were recorded as the reciprocals of the highest dilutions producing positive results.

ELISA

The ELISAs were performed in 96 well immunoassay plates (Nunc, Roskilde, Denmark), and optimal working dilutions of the reagents were determined by chessboard titration. Throughout the assays, reagent volumes of 100 μl were used, the diluent for reagents was phosphate-buffered saline (PBS), pH 7·2, containing

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10% foetal calf serum (State Vaccine Institute, Cape Town, South Africa), incubations were performed for 1 h at 37 °C, wells were post-coated with 200 μl PBS containing 2% bovine serum albumin (Calbiochem, La Jolla, Ca, USA) and plates were washed thrice with PBS containing 0·1% Tween 20 (Merck, Darmstadt, Germany), unless specified otherwise.

IgM-capture ELISA.

The presence of IgM antibody to CCHF virus was demonstrated by IgM-capture ELISA using rabbit anti-CCHF HRPO conjugate as described previously for sheep and cattle sera [20] and adapted for testing human sera by coating the plates overnight at 4 °C with μ-chain specific anti-human IgM (Zymed) diluted 1/1000 with carbonate buffer, pH 9·6. After post-coating the plates were washed, and test sera were added to the wells in doubling dilutions from 1/200 upwards. The plates were incubated, washed and CCHF antigen diluted 1/200 was added to the wells. After further incubation and washing, rabbit anti-CCHF HRPO conjugate, diluted 1/1000, was added to the wells and the plates incubated. After further washing, the substrate, 2,2′-azino-di-(3-ethyl-benzthiazoline-6-sulphonate) (ABTS) (Kirkgaard and Perry Laboratories, Gaithersburg, MD, USA), was added and the plates held at room temperature (22 °C) for 30 min in the dark. The results were determined by reading the optical density (OD) at 420 nm on a Multiskan spectrophotometer (Flow Laboratories Inc., McLean, VA, USA). Specimens were considered to be IgM antibody positive if the absorbance in the sample wells was at least twice the value of that for human negative control serum.

IgG sandwich ELISA.

The IgG antibody response was measured by sandwich ELISA in which the plates were coated overnight at 4 °C with monoclonal antibody 6E5 diluted 1/2000 in carbonate buffer, pH 9·6. After post-coating, antigen diluted 1/200 was added to wells, the plates were incubated, washed and test sera were added in doubling dilutions from 1/100 upwards. The plates were incubated, washed and anti-human IgG HRPO conjugate (Zymed) was added at a dilution of 1/1000. After further incubation and washing, substrate was added, allowed to react and the results recorded as above.

CELISA.

Total antibody activity was determined in a CELISA in which test sera competed with coating antibody for binding of antigen, with immobilized antigen being detected by anti-CCHF HRPO conjugate as described previously for sheep and cattle sera [20]. Sera were tested in doubling dilutions from 1/10 upwards and considered positive if the OD reading was $\leqslant 50\%$ of that produced by human negative control serum [21].

RESULTS

Confirmation of the diagnosis in CCHF patients

Altogether, 2998 specimens from 1576 suspected cases of viral haemorrhagic fever were examined from January 1980–February 1992 inclusive. Among the 101 cases of CCHF encountered, the laboratory diagnosis was confirmed in 67

instances by isolation of virus from one or more serum samples taken during the first 13 days of illness, or from liver samples taken after death in fatal infections, together with the demonstration by IF of seroconversion or rising antibody titres in survivors of the disease. In a further 25 patients the laboratory diagnosis was based on the demonstration of seroconversion, or a $\geqslant$ 4-fold increase in IF antibody titres. In the remaining nine patients the laboratory diagnosis depended on the demonstration by IF of IgM antibody activity (at titres of 64–256) in single sera or repeat samples collected at close intervals, supported by a history of potential exposure to infection, the nature of the illness experienced by the patients, and other laboratory data (thrombocytopenia, haemostatic derangement and raised serum levels of transaminases and bilirubin).

In presenting the results of the serologic tests (Figs 2, 3), distinction is made between fatal and non-fatal disease since the immune response differs markedly with the outcome of infection [11]. A total of 25 patients died. The deaths of 23 patients between days 5–14 of illness were ascribed directly to the effects of CCHF infection. Two patients survived the acute illness and died later from other causes, and hence the results obtained on their sera are included with the findings on the remaining 76 survivors of the disease. One of these two patients was not initially recognized as a case of CCHF and had an operation for the drainage of cerebral haemorrhage. He removed a ventriculo-peritoneal bypass drainage tube himself and died on day 27 of illness from purulent meningitis associated with *Staphylococcus aureus* infection apparently acquired through the surgical wound [22]. Both IgM and IgG antibody responses were demonstrated in sera tested retrospectively, but no lesions or virological evidence of active CCHF infection were found at autopsy. The second patient had suffered for years from chronic bronchitis, coronary heart disease and hypertension for which he had frequently been treated in hospital; he appeared to recover from CCHF infection which had been confirmed by isolation of virus from serum and demonstration of an immune response, but died on day 24 of illness from pneumonia and a pseudomonas septicaemia.

It must also be taken into account that 18 patients, 4 of whom died in the acute stage of illness plus 14 survivors, received intravenous treatment with one or more 250 ml units of immune plasma collected from recovered patients. The plasma units lacked IgM antibody demonstrable by IF or had minimal titres of 8, but had IF IgG titres of 256–4096, and neutralizing titres of 32–512 (cell culture fluorescent-focus reduction) [11, 12]. Recipients of immune plasma acquired demonstrable levels of serum IF IgG antibody activity within 24 h of treatment. However, there was no corresponding immediate appearance of demonstrable IF IgM antibody activity in their sera, and treated patients seemed to acquire IgM antibody only from endogenous response at the same stage of illness as their untreated counterparts.

IF antibody response

No antibodies were demonstrable by IF in any sera taken during the first 3 days of illness (Figs 2*a*, 3*a*). Excluding those persons who received immune plasma (treated patients), only about 10% of survivors (range 1/12–2/13) tested on days 4 and 5 of illness had developed demonstrable IF IgM and/or IgG antibody

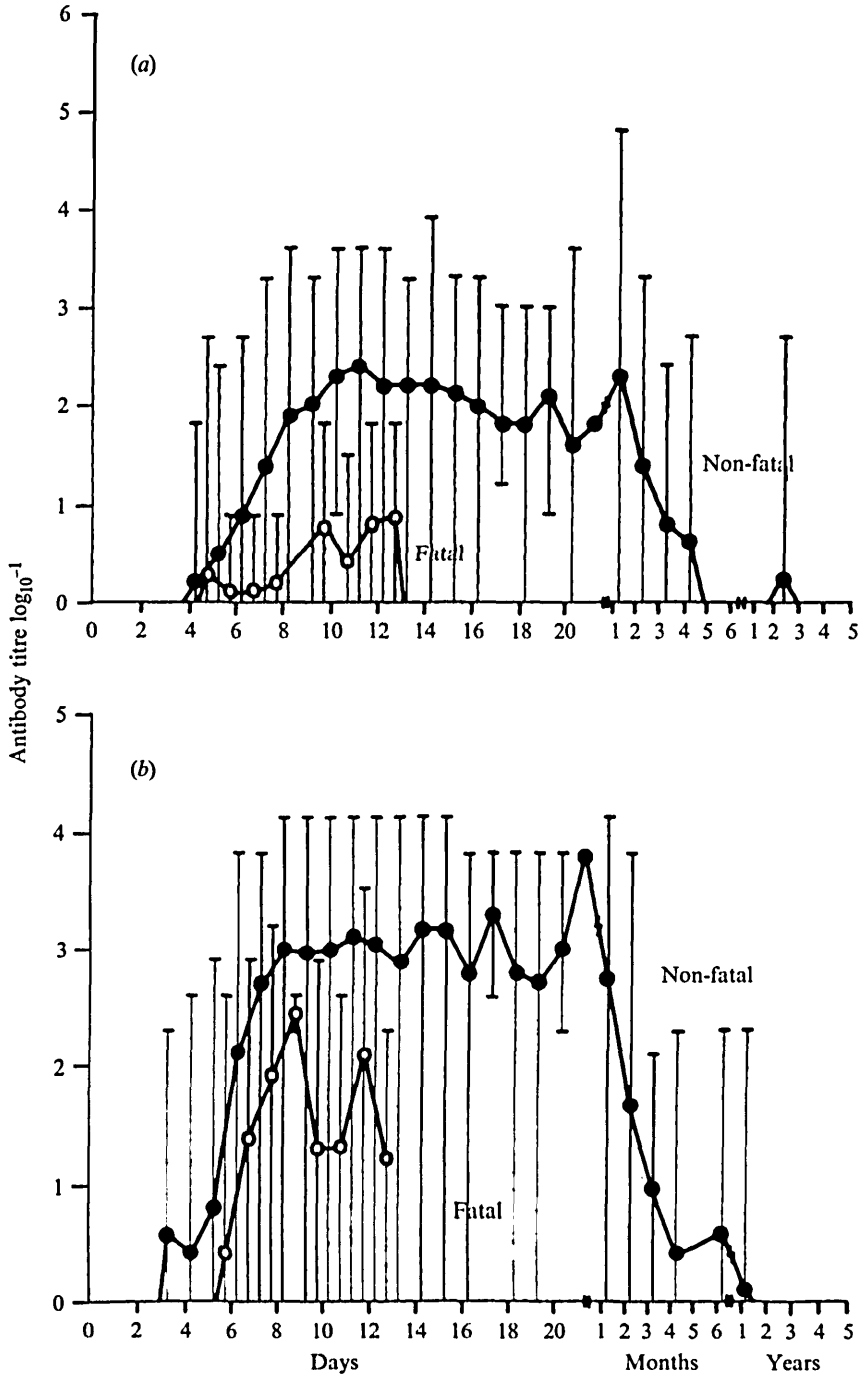

Fig. 2. IgM antibody response in Crimean–Congo haemorrhagic fever patients detected by (*a*) immunofluorescence and (*b*) IgM-capture enzyme-linked immunoassay. Curves show geometric mean antibody titres and range at the indicated intervals after onset of illness.

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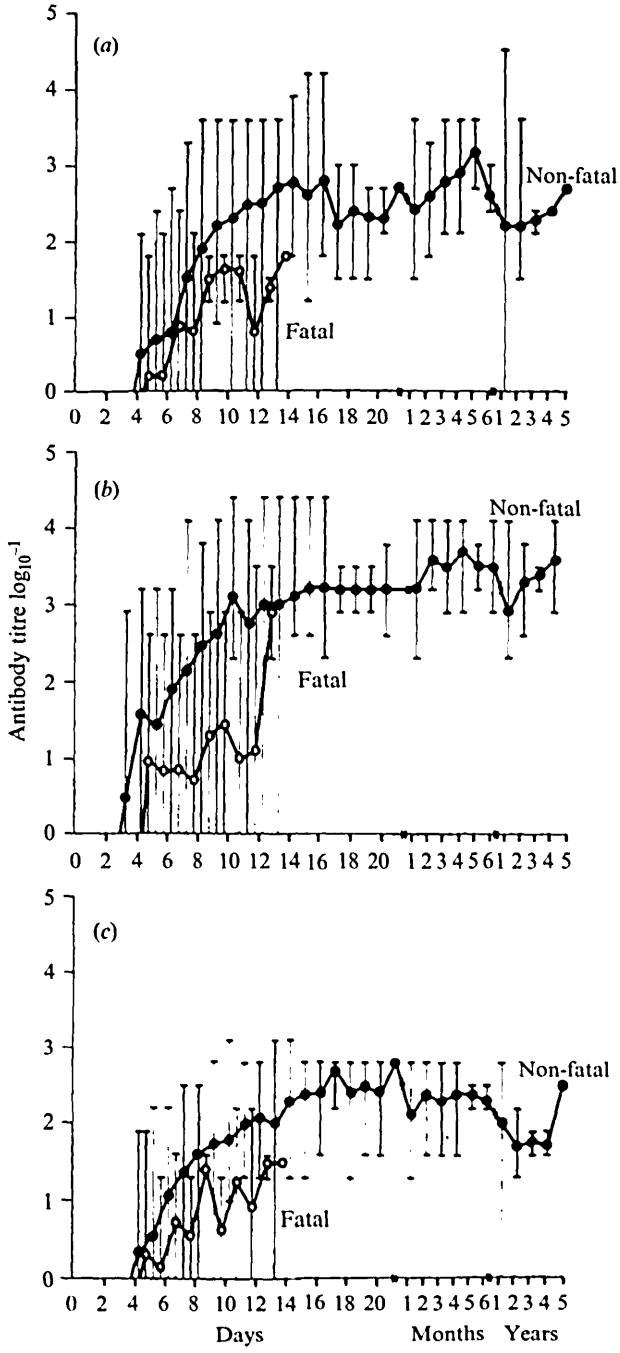

Fig. 3. IgG antibody response in Crimean–Congo haemorrhagic fever patients detected by (*a*) immunofluorescence; (*b*) IgG sandwich enzyme-linked immunoassay and (*c*) total antibody activity demonstrated by competition enzyme-linked immunoassay. Curves show geometric mean antibody titres and range at the indicated intervals after onset of illness.

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activity. By day six 11/17 untreated survivors tested had IgM antibody and 6 of them had IgG antibody demonstrable by IF. On day 7 IgG and/or IgM antibody was detected in 25/30 untreated survivors and this rose to 30/32 untreated survivors by day 8. All of 32 untreated survivors tested on day 9 had demonstrable IF IgM and IgG titres, but in a single patient IgG antibody continued to fluctuate between undetectable levels and a minimal titre of 8 during the first 3 weeks of illness, and in another patient IgM antibody behaved in the same way.

In 57 survivors of the acute illness, plasma-treated and untreated, the maximum IF IgG antibody titres were recorded towards the end of the second week of illness, particularly on day 11, or during the third week, while in the remaining survivors there was a late rise in antibody level to maximum titre during the second to fifth month after onset of illness. Maximum IgG antibody titres recorded were nearly all in the range 256–4096, but titres of 8192 and 16384 were detected in two patients, while in one patient there was a late increase in IgG antibody level to a titre of 32768 at 16 months. Maximum IgG titres of 16–128 were recorded in a few survivors who were tested only during the first 2 weeks of illness. There was generally a two- to fourfold reduction in IgG antibody level within days of the maximum titre being attained, but thereafter levels declined gradually or stabilized, and one patient bled at 59 months still had a titre of 512. The only anomaly was that one patient lacked demonstrable IF IgG antibody on one occasion when tested a year after the onset of his illness.

The onset and early course of the IF IgM antibody response in all survivors of infection, plasma-treated and untreated, resembled the pattern of the IgG response in untreated survivors, with a slight tendency for IgM to become demonstrable a day or so earlier than IgG antibody in untreated patients, although the reverse also occurred in some individuals. As with IgG antibody, the attainment of maximum IgM antibody levels followed a bimodal pattern, with the highest titres in most patients being recorded towards the end of the second or into the third week of illness, but with a few peak titres being recorded 2–4 months after onset of illness. However, IgM antibody activity had declined to undetectable or minimal levels in most patients by 4 months, and thereafter IgM antibody was detected by IF on a single occasion in a patient who had an anomalous titre of 512 two years after the onset of illness (Fig. 1*a*). The maximum IF IgM antibody titres recorded in patients were generally two- to fourfold lower than the peak IgG titres in the same individuals. Maximum titres of 32 were recorded in two patients who were tested only during the first 2 weeks of illness; otherwise peak titres fell into the range 64–4096, except for titres of 8192 recorded in two patients.

Among the 23 patients who succumbed during the acute illness, the 4 who received immune plasma therapy all acquired IgG antibody activity demonstrable by IF at maximum titres of 16–256, and 3 of these had weak IgM antibody activity, fluctuating between minimal titres of 8 and undetectable levels. Among the 19 untreated patients, IF antibody response was detected in only 4 individuals who were admitted to hospital at an advanced stage of illness and died within 48 h. Three had peak IgG titres of 16–64 and IgM titres of 64–512, while the fourth had only IgM antibody activity at a titre of 64.

It was possible to detect IgG and/or IgM IF antibody responses at an earlier stage in some patients by lowering the starting dilution of serum to 1/4, but in

many instances non-specific fluorescence interfered with the interpretation of results at this dilution, possibly because patients had been treated with high doses of fluorescing drugs such as tetracycline. Hence the starting dilution of 1/8 was adopted for routine use. Consistent results could be obtained in IgG IF tests with minimum incubation periods of 20 min for sera and conjugates on antigen slides at 37 °C. Although positive results could be obtained in IgM IF tests after 1·5 or 3 h incubation of sera on slides, results were most consistent and titres highest after overnight incubation. No overt problems were encountered with rheumatoid factor in CCHF patients, and pre-treatment of sera made little or no difference to the interpretation of IgM IF tests, but it was discovered that patients with severe or fulminant hepatitis A (and suspected to be suffering from viral haemorrhagic fever) frequently had non-specific IgM activity in IF tests, with titres of up to 32768 despite pre-treatment of sera. Sera from patients with malaria, or from malaria-affected areas, often exhibited non-specific fluorescence at low titre in both IgG and IgM tests, but it was not established whether this was possibly associated with the use of anti-malarial drugs, or with raised serum immunoglobulin levels.

The inclusion of the extra staining and washing steps involved in the biotin-streptavidin procedure added 40–60 min to the time required for IF tests, and resulted in increases of two- to eightfold in IgG and IgM titres in many instances (data not shown), but rendered IgG antibody detectable one day earlier than the routine IF in only 1/39 patients tested. In IgM tests, however, the use of the biotin-streptavidin procedure resulted in the demonstration of antibody response in a fatal (plasma-treated) infection in which only IgG antibody had been detected in the routine IF tests, and in 6/35 survivors of infection tested IgM antibody became detectable one to 3 days earlier than by routine IF. The antibodies nevertheless became detectable no earlier than day 5 of illness in 2 patients, and on days 6 and 7 in the other 4 instances.

ELISA antibody response

Antibody levels recorded in IgM-capture and IgG sandwich ELISAs (Figs. 2*b*, 3*b*) were much higher than in the corresponding IF tests, with maximum titres recorded in most survivors of infection falling in the range 200–25600. Moreover, IgM antibody was detected in 4 fatal infections in which it had not been demonstrable by IF, and in 15/78 survivors of the acute disease IgM antibody was demonstrable 1–3 days earlier than by IF, being recorded as early as day 3 of illness in 2 patients. However, in a further 14 non-fatal infections IgM antibody was detected earlier by IF than by ELISA. Similarly, IgG antibody was detected by ELISA in 3 fatal infections in which it had not been demonstrable by IF, and in 10 non-fatal infections IgG antibody was detected earlier by ELISA, as early as day 3 in one instance, but in a further 11 survivors the antibody response was detected earliest by IF. The CELISA produced lower antibody titres than either the IgM-capture or IgG sandwich ELISAs, but the results obtained (Fig. 3*c*) were generally consistent with those obtained in IF IgG tests. Maximum titres recorded in individual survivors of infection fell into the range 80–640.

Results obtained in ELISAs were found to be most consistent if plates were coated freshly before use, rather than coated earlier and stored at −70 °C. The

sensitivities of the IgM-capture and IgG sandwich ELISAs could be improved slightly by using a 1/50 starting dilution for test sera, but this advantage was outweighed by the tendency to obtain occasional false-positive results in non-infected persons at the lower dilution.

DISCUSSION

In brief, the findings were that routine IF tests detected IgM and IgG antibodies to CCHF virus in an increasing proportion of patients from the fourth day of illness onwards, and that all persons who survived the disease had antibodies demonstrable by IF on day 9. Antibody was demonstrable by IF in only 4/19 patients who died from the disease and had not received immune plasma. The biotin-streptavidin IF technique offered no advantage over the standard IF test for the early detection of IgG antibody, but demonstrated higher titres and detected IgM antibody earlier in 6/35 of the patients tested. The IgM-capture and IgG sandwich ELISAs demonstrated higher antibody titres than did IF, and each detected a response in four fatal infections in which no response could be found by IF. Among survivors of the disease, the IgM and IgG ELISAs detected an immune response at an earlier stage of infection than did IF tests in 10 and 15 patients respectively, but the reverse was true in similar numbers of patients (11 and 14 respectively). The failure of the ELISAs to produce positive results on occasion with sera which had low IF titres, was possibly related to the higher starting dilutions used in these tests. The CELISA, in which sera were tested at a starting dilution of 1/10, produced lower titres than did the IgM and IgG ELISAs, but yielded results which were in close agreement with the findings in IF tests.

Early recognition of CCHF usually occurs only when patients or clinicians are alert to incidents constituting specific exposure, as in nosocomial infections or following the occurrence of a bite by a *Hyalomma* tick vector of the virus, so that comparatively few specimens are submitted for the diagnosis of the disease during the first 3 days of illness before antibodies become demonstrable (Fig. 1). There is an increasing probability of arriving at a rapid serologic diagnosis on specimens submitted from day 4 of illness onwards, and the detection of IgG and/or IgM antibody by IF is virtually certain by day 9 in non-fatal infections. However, it cannot be assumed that the initial history which accompanies specimens is accurate with respect to the date of onset of illness, and it should be routine practice to screen all sera for antibody to the virus.

We find it most convenient to screen sera for IgG antibody activity by IF on arrival, and often a positive result is obtained which allows a tentative diagnosis to be reported in little more than an hour of receiving the specimen. At the same time, confirmatory IgM IF tests are prepared for overnight incubation, cell cultures and laboratory animals are inoculated for isolation of the virus, and plates are coated in preparation for ELISAs on the following day. Use of the biotin-streptavidin IF procedure may increase the chances of early detection of IgM antibody, but the ELISAs for IgG and IgM antibody are as likely as not to increase the rapidity with which a diagnosis is attained. Further improvement in making a rapid diagnosis early in the course of the disease, before antibody becomes demonstrable, must depend on the refinement of virological methods,

such as the detection of virus antigen in clinical specimens, daily examination of replicate cell cultures by immunofluorescence for isolation of virus, or demonstration of viral nucleic acid by reverse transcription and the polymerase chain reaction (currently under investigation).

Because no antibody response was detected by any of the serological techniques in the majority of the patients who succumbed to the disease, or the response was at best delayed and weak in fatal infections (Figs. 2, 3), it emerged from our observations that the demonstration of rising antibody titres constituted a favourable prognostic sign. As a corollary, we found that failure to detect antibodies on day 9 of illness in patients who were not moribund at that stage, rendered a diagnosis of CCHF extremely unlikely.

Nairoviruses generally induce weaker neutralizing antibody responses than do members of the other genera of the family Bunyaviridae [23], and it was previously shown that this was true for CCHF infection [11]. It can be added from the present observations that the appearance of circulating antibody did not correlate directly with the clearance of viraemia, and virus could be isolated with facility from patients' sera up to day 13 of illness despite the presence of antibody at high titre as demonstrated by any of the methods used here, and despite the fact that survivors felt better and their clinical pathology values such as platelet counts and serum transaminase levels improved from about day 10 of illness onwards. Nor did the administration of immune plasma appear to eliminate demonstrable viraemia or improve the chances of surviving the disease. On the other hand, it must be noted that individual patients received from 1–9 units of plasma which were not uniform with respect to neutralizing antibody potency, and were treated at various stages of illness up to and including terminal coma, so that no firm conclusions can be drawn on the efficacy of the treatment.

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