INDIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETECTION OF IgG ANTIBODIES AGAINST COXSACKIE B VIRUSES

GILLIAN HANNINGTON, J. C. BOOTH, C. N. WIBLIN* AND H. STERN

*Department of Medical Microbiology, St. George's Hospital Medical School, London
SW17 0RE and *Vaccine Research and Production Laboratory, PHLS Centre for
Applied Microbiology and Research, Porton Down, Salisbury, Wilts SP4 0JG*

SUMMARY. In tests for IgG antibodies against Coxsackie B viruses in man, the enzyme-linked immunosorbent assay (ELISA) was essentially group-specific and, unlike the type-specific neutralisation test, usually failed to detect rises in antibody titre in paired, acute and convalescent, sera. However, in rabbits immunised against Coxsackie B viruses, ELISA demonstrated both group- and type-specific antibody responses. The lack of type-specificity of ELISA in man is probably because repeated infection with enteroviruses—echoviruses and Coxsackie A as well as Coxsackie B—results in masking of the type-specific antibody response by group-specific antibody.

INTRODUCTION

Coxsackie B viruses are important causes of human disease; the more severe infections involve the central nervous system and the heart (Grist, Bell and Assaad, 1978). Diagnosis often depends upon detecting specific antibodies because excretion of virus commonly ceases soon after onset of symptoms (Bell and Grist, 1968). The most reliable serological technique has been the neutralisation test, which detects type-specific antibody responses. However, the neutralisation test is laborious, time consuming and slow and a quicker and simpler technique is needed for diagnostic purposes.

Since its introduction by Engvall and Perlmann (1971) and Van Weemen and Schuurs (1971), the enzyme-linked immunosorbent assay (ELISA) has proved to be a highly sensitive and versatile technique in viral serology, and is capable of detecting type-specific and group-specific antibodies in hyperimmune rabbit sera prepared against various enteroviruses (Herrmann, Hendry and Collins, 1979; Katze and Crowell, 1980a). We have investigated the specificity of the indirect ELISA technique for detecting Coxsackie-B-virus antibodies in rabbit and human sera, with a view to evaluating its usefulness as a diagnostic test.

Received 18 Apr. 1983; accepted 25 May 1983.

MATERIALS AND METHODS

Viruses. Coxsackievirus types B1–B5 were obtained from Dr G. Cambridge, Department of Virology, Hammersmith Hospital, London. Stocks were prepared in continuous African green monkey kidney cells (Vero) and stored at -70°C .

Preparation of purified antigens for the ELISA test. Confluent monolayers of Vero cells in roller bottles were washed three times with phosphate buffered saline (PBS), pH 7.4, reincubated in Eagle's minimal essential medium (MEM) supplemented with glutamine 0.03% for 72 h, and infected with coxsackievirus, *c.* 10^9 plaque-forming units (pfu)/roller bottle (input multiplicity = 2 pfu/cell). After adsorption for 1 h at 37°C , the cultures were refed with MEM supplemented with glutamine 0.03% and tryptose-phosphate 1% and incubated at 37°C for 36–60 h, until 3–4+ CPE developed. The supernates were discarded and the infected cells harvested before they had spontaneously detached from the glass by shaking with sterile glass beads. The infected cells were collected into MEM and deposited by centrifugation at 4000 rpm for 1 h. They were resuspended in a small volume of MEM and ruptured by freezing and thawing three times. The cell debris was removed by spinning at 2500 rpm for 30 min and the supernate, containing the virus particles, was stored in small volumes at -70°C until required; the infectivity titres were *c.* 10^7 TCD₅₀/0.05 ml. For purification, 2-ml amounts were mixed with 0.2 ml of a 10% solution of sodium lauryl sulphate (Brown *et al.*, 1976) and centrifuged in a preformed linear sucrose gradient (sucrose 15–45% w/w, in PBS) overlying a cushion of sucrose 60%, for 2 h at 40 000 rpm in the Beckman SW41 rotor. One-ml fractions were harvested, and 90% of the infectivity was recovered within a 1–4 fraction band at the centre of the gradient, well separated from the peak of material dissolved in detergent at the top of the gradient as judged by absorbance measurements at 260 and 280 nm. The purified virus was stored at -70°C , and was subsequently used as antigen for the ELISA test and for the preparation of immune sera in rabbits; infectivity titres ranged from $10^{5.5}$ – $10^{7.9}$ TCD₅₀/0.05 ml.

Control antigens were prepared in the same way from uninfected Vero cell cultures.

Preparation of immune antisera. New Zealand white rabbits, without pre-existing antibodies against Coxsackie B viruses, were given 0.3 ml of purified virus intravenously and a second dose 3 weeks later; they were bled after a further 3 weeks.

Human sera. Serum specimens were available from 50 adults, aged 31–72 years (mean 52 years), who had been examined previously for neutralising antibodies to Coxsackie B virus types 1–5 (Griffiths, Hannington and Booth, 1980); they were stored at -20°C . The sera included seven samples without detectable antibody to any of the five viruses, 12 that contained type-specific antibodies reacting with only a single serotype, and 31 with neutralising antibodies for two or more serotypes. Paired serum specimens were also available from another four patients; they showed a reproducible four-fold or greater rise in neutralising antibody titre against at least one of the virus serotypes.

Neutralisation technique. Tests were done in Microtitre plates, with Vero cells, as previously described (Griffiths *et al.*, 1980).

ELISA technique. This was performed as described by Booth *et al.* (1979), except that viral antigen was fixed to the Microtitre plates by drying at room temperature overnight followed by treatment with formalin 10% for 20 min. The absorbance value (E_{400}) for each serum sample was calculated as the mean of readings of duplicate wells minus the "background" activity in the test; the latter was the mean of the readings from duplicate wells incubated with diluent instead of serum. Serum antibody titration end-points were the lowest dilution of the serum with an E_{400} reading of 0.2; control wells without antigen or with serum specimens without specific antibodies gave readings <0.2 .

Pre-titration of antigens and ELISA conjugates. The conjugates (sheep anti-human IgG and sheep anti-rabbit immunoglobulin, coupled with alkaline phosphatase) and the five purified virus antigens were titrated in parallel in a single chessboard test. Microtitre plates were coated with antigen, in doubling dilutions from 1 in 10 to 1 in 80, and treated with a 1 in 300 dilution of a pool of human sera or rabbit antisera, prepared as described above, containing neutralising antibodies to all five Coxsackie B virus serotypes. Conjugate was then added, in serial two-fold dilutions from 1 in 50 to 1 in 400 in chessboard fashion, and the ELISA test was completed in the usual way. The working dilution for each antigen was taken as the dilution that gave an E_{400}

reading of 1.0 with a 1 in 200 dilution of the conjugate; the dilution factors for the five virus serotypes ranged from 1 in 10 to 1 in 80.

RESULTS

Tests with immune rabbit antisera

The neutralisation test showed that the five immune rabbit sera prepared against Coxsackie types B1–5 were type specific (table I). The antibody titres ranged from 64–2048, and there were no cross-reactions with the heterotypic antigens. By ELISA, the homotypic antibody titres were 3- to 35-fold higher than by the neutralisation test, and all five sera showed extensive cross-reactions with heterotypic antigens, although the cross-reacting antibody titres were 2- to 8-fold lower than the type-specific antibody titres. None of the rabbit pre-immunisation sera had detectable Coxsackie B virus antibodies, either by ELISA or by the neutralisation test. None of the sera reacted significantly with the control antigen, even when tested at a dilution of 1 in 100.

TABLE I

ELISA and neutralising antibody titres in rabbits immunised against Coxsackie B viruses, types 1 to 5

Test virus	ELISA (and neutralising) antibody titres of antisera prepared against virus serotype				
	B1	B2	B3	B4	B5
B1	25600 (2048)	800 (<8)	400 (<8)	3200 (<8)	800 (<8)
B2	1600 (<8)	1600 (64)	6400 (<8)	12800 (<8)	800 (<8)
B3	3200 (<8)	400 (<8)	25600 (1024)	1600 (<8)	800 (<8)
B4	1600 (<8)	800 (<8)	5200 (<8)	25600 (1024)	400 (<8)
B5	3200 (<8)	800 (<8)	3200 (<8)	6400 (<8)	3200 (1024)

Homologous antibody titres are shown in **bold** type.

Tests with human sera

Paired sera from four patients that showed four-fold or greater rises in neutralising antibody levels to one or more of the Coxsackie B virus serotypes were titrated for antibody by ELISA (table II). All eight sera contained antibodies against all five coxsackieviruses but rising titres were not demonstrated, nor were there significant differences in the antibody levels, in each serum specimen, against the five virus serotypes.

Single serum specimens collected from 50 adult patients were examined for coxsackievirus antibodies. By ELISA, all 50 sera had antibodies against all five virus serotypes. However, only 43 had neutralising antibodies against one or more of the viruses; the seven negative sera were also without Coxsackie B6 neutralising antibody. When the neutralising antibody titres of the 50 sera were compared with the ELISA- E_{400} readings, which are proportional to the ELISA-antibody titres, for each serotype in turn, significant correlations were not demonstrated; r values ranged from 0.20–0.32 ($p > 0.1$). Similar findings were obtained when the neutralising antibody titres against the five virus serotypes were compared with one another; e.g., comparison of Coxsackie B2 and B5 antibody titres gave r values of 0.17–0.47 ($p > 0.1$).

TABLE II

ELISA and neutralising antibody titres against Coxsackie B viruses, types 1 to 5, in acute (A) and convalescent (B) sera from four adult patients

Patient no. and serum	ELISA (and neutralising) antibody titres obtained against virus serotypes				
	B1	B2	B3	B4	B5
1 { A	1400 (<8)	1400 (32)	1800 (<8)	1400 (8)	1400 (<8)
1 { B	1800 (<8)	2400 (64)	1800 (<8)	1600 (64)	1300 (<8)
2 { A	4500 (64)	10000 (<8)	4500 (64)	3600 (8)	4000 (<8)
2 { B	4500 (64)	8000 (128)	5600 (64)	4000 (8)	4000 (8)
3 { A	4000 (16)	9000 (8)	2200 (32)	1400 (16)	3600 (64)
3 { B	4500 (64)	8000 (<8)	3600 (64)	1800 (8)	6000 (128)
4 { A	5600 (128)	11200 (16)	8900 (<8)	11200 (> 1024)	14100 (128)
4 { B	10000 (> 1024)	11200 (256)	10000 (<8)	14100 (> 1024)	12600 (512)

Four-fold or greater increases in titres between acute and convalescent sera are shown in **bold type**.

(fig. a). In contrast, the ELISA- E_{400} readings for the five virus serotypes showed a highly significant correlation; e.g., Coxsackie B2 and B5 antibody titres gave r values of 0.90–0.93 ($p=0.001$) (fig. b). E_{400} readings were within the range 0.21–1.17 in tests with the viral antigens, and 0.01–0.16 with the control antigen (mean 0.04, standard deviation 0.03).

The seven sera without neutralising antibodies contained ELISA antibodies against all five Coxsackie B virus serotypes, and the E_{400} values (0.36–0.57) were within the middle range obtained for the group of 50 sera.

FIG.—Correlation of (a) neutralising antibody titres and (b) ELISA- E_{400} values obtained against Coxsackie B2 and Coxsackie B5 viruses in 50 human sera.

DISCUSSION

The neutralisation test proved to be, as expected, type-specific in its reactivity but the ELISA test, even with purified virus particles, was essentially group-specific. With the rabbit immune sera, however, there was evidence that ELISA was detecting both group-specific and type-specific antibodies, because the antibody titre against the homologous virus was invariably higher than the titres obtained with the heterologous viruses. This confirms the findings of Herrmann *et al.* (1979) and Katze and Crowell (1980a) who demonstrated similar group- and type-specific reactions in indirect-ELISA tests with rabbit antisera and purified Cocksackie B viruses.

Other techniques, such as complement fixation, gel diffusion and indirect immunofluorescence, are also mainly group-reactive in tests for coxsackievirus antibodies (Schmidt, Dennis and Lennette, 1967; Chaudhary and Westwood, 1970). Group-enteroviral antigens appear to be exposed on naturally occurring incomplete virus particles and on complete virus particles that have been denatured by heating (Schmidt *et al.*, 1963). Heating results in the loss of the VP4 polypeptide from the virus particle (Breindl, 1971; Maizel, Phillips and Summers, 1967) or in gross conformational re-arrangement of the proteins of the virus capsid (Lonberg-Holm and Yin, 1973). There is evidence that similar disturbances, with exposure of group-antigenic determinants, occur when Cocksackie B virus particles adsorb on to the surface of Microtitre plates (Katze and Crowell, 1980a and b).

Although ELISA detected group- and type-specific antibody responses in the rabbit immune sera, it failed to recognise rising titres of antibody in the paired, acute and convalescent, human sera. Unlike the neutralisation test, ELISA showed, in the paired sera, only the presence of group-reactive antibody which masked the type-specific response. Human sera are more likely than animal immune sera to possess excess group antibody because of the repeated exposure of man to naturally occurring infections with coxsackieviruses (Schmidt *et al.*, 1967); all the sera examined in the present investigation were from adults over 31 years old. That most of the antibody present in these sera was group-reactive was confirmed by the highly significant correlation between the ELISA-antibody titres obtained against the different viruses, in contrast to the lack of such correlation for the neutralising antibody titres. ELISA may detect type-specific antibody responses more readily in children, but 20% or more of children have been exposed to infection with one or more coxsackievirus serotypes by 4 years of age (Cramblett *et al.*, 1964; Doerr *et al.*, 1977). Seven of the 50 adult sera examined showed a group-specific ELISA-antibody response against the Cocksackie B viruses but were without detectable coxsackievirus neutralising antibodies. This is almost certainly due to the wide sharing of group antigens with other members of the enterovirus group, which includes echoviruses and Cocksackie A viruses (Halonen, Rosen and Huebner, 1959; Schmidt *et al.*, 1965 and 1967).

This masking effect of pre-existing enteroviral group antibody, preventing the demonstration of rising titres of either group- or type-specific antibody following infection with another virus of the group, presents problems in the use of ELISA for the diagnosis of recent infection. An alternative to examining for rising levels of IgG antibody is to test for virus-specific IgM antibody in a single serum specimen. A Cocksackie B virus, or any enterovirus, group-IgM response would facilitate the

development of a reliable ELISA test for recent infection, and El-Hagrassy, Banatvala and Coltart (1980) have described a Coxsackie B virus IgM-antibody-capture technique using a polyvalent mixture of type-specific antigens to give group reactivity. However, recent work from our laboratory suggests that the use of only one of the Coxsackie B viruses as antigen in the indirect-ELISA test is adequate to detect a group-enteroviral IgM-antibody response in sera from adults (G. Hannington, unpublished observations).

We are indebted to the British Heart Foundation for generous financial support, and to Gerry Wilkins and Joyce Hacker of the PHLS at Porton for the preparation of the antigen concentrates.

REFERENCES

- BELL, E. J. AND GRIST, N. R. 1968. Coxsackie virus infections in patients with acute cardiac disease and chest pain. *Scottish Medical Journal*, **13**, 47–51.
- BOOTH, J. C., HANNINGTON, G., AZIZ, T. A. G. AND STERN, H. 1979. Comparison of enzyme-linked immunosorbent assay (ELISA) technique and complement-fixation test for estimation of cytomegalovirus IgG antibody. *Journal of Clinical Pathology*, **32**, 122–127.
- BREINDL, M. 1971. The structure of heated poliovirus particles. *Journal of General Virology*, **11**, 147–156.
- BROWN, F., WILD, T. F., ROWE, L. W., UNDERWOOD, B. O. AND HARRIS, T. J. R. 1976. Comparison of swine vesicular disease virus and Coxsackie B5 virus by serological and RNA hybridization methods. *Journal of General Virology*, **31**, 231–237.
- CHAUDHARY, R. K., AND WESTWOOD, J. C. N. 1970. Detection of cross-reactions by immunofluorescence within the picornavirus group. *Canadian Journal of Microbiology*, **16**, 601–604.
- CRAMBLET, H. G., MOFFET, H. L., BLACK, J. P., SHULENBERGER, H., SMITH, A. AND COLONNA, C. T. 1964. Coxsackie virus infections in clinical laboratory studies. *Journal of Pediatrics*, **64**, 406–414.
- DOERR, H. W., SCHWEISS, H. D., HENNINGER, K. AND SOCHANIK, H. 1977. Simple mathematical deductions in the seroepidemiology of viral infections. II (Para) myxoviruses (measles, mumps, influenza B), rubella, enteroviruses (polio, Coxsackie B), adenoviruses, and *Mycoplasma pneumoniae*. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene*. I. Abt. Orig., **A238**, 165–176.
- EL-HAGRASSY, M. M. O., BANATVALA, J. E. AND COLTART, D. J. 1980. Coxsackie B virus specific IgM responses in patients with cardiac and other diseases. *Lancet*, **2**, 1160–1162.
- ENGVALL, E. AND PERLMANN, P. 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry*, **8**, 871–874.
- GRIFFITHS, P. D., HANNINGTON, G. AND BOOTH, J. C. 1980. Coxsackie B virus infections and myocardial infarction. Results from a prospective, epidemiologically controlled study. *Lancet*, **1**, 1387–1389.
- GRIST, N. R., BELL, E. J. AND ASSAAD, F. 1978. Enteroviruses in human disease. *Progress in Medical Virology*, **24**, 114–157.
- HALONEN, P., ROSEN, L. AND HUEBNER, R. J. 1959. Homologous and heterologous complement fixing antibody in persons infected with ECHO, Coxsackie and Poliomyelitis viruses. *Proceedings of the Society for Experimental Biology and Medicine*, **101**, 236–241.
- HERRMANN, J. E., HENDRY, R. M. AND COLLINS, M. F. 1979. Factors involved in enzyme-linked immunoassay of viruses and evaluation of the method for identification of enteroviruses. *Journal of Clinical Microbiology*, **10**, 210–217.
- KATZE, M. G. AND CROWELL, R. L. 1980a. Indirect enzyme-linked immunosorbent assay (ELISA) for the detection of Coxsackievirus group B antibodies. *Journal of General Virology*, **48**, 225–229.
- KATZE, M. G. AND CROWELL, R. L. 1980b. Immunological studies of the group B

- Coxsackieviruses by the sandwich enzyme-linked immunosorbent assay (ELISA) and immunoprecipitation. *Journal of General Virology*, **50**, 357–367.
- LONBERG-HOLM, K. AND YIN, F. H. 1973. Antigenic determinants of infective and inactivated human rhinovirus type 2. *Journal of Virology*, **12**, 114–123.
- MAIZEL, J. V., PHILLIPS, B. A. AND SUMMERS, D. E. 1967. Composition of artificially produced and naturally occurring empty capsids of poliovirus type 1. *Virology*, **32**, 692–699.
- SCHMIDT, N. J., DENNIS, J., FROMMHAGEN, L. H. AND LENNETTE, E. H. 1963. Serologic reactivity of certain antigens obtained by fractionation of Coxsackie viruses in cesium chloride density gradients. *Journal of Immunology*, **90**, 654–662.
- SCHMIDT, N. J., DENNIS, J. AND LENNETTE, E. H. 1967. Antibody responses of rhesus (*Macaca mulatta*) monkeys experimentally infected with Coxsackieviruses of group B and group A, type 9. II Heterotypic antibody responses to echoviruses, polioviruses and reovirus type 1. *Journal of Immunology*, **98**, 1060–1066.
- SCHMIDT, N. J., DENNIS, J., LENNETTE, E. H., HO, H. H. AND SHINOMOTO, T. T. 1965. Antibody responses of rhesus (*Macaca mulatta*) monkeys experimentally infected with Coxsackieviruses of group B and group A, type 9. I Antibody responses within the Coxsackievirus group. *Journal of Immunology*, **95**, 54–69.
- VAN WEEMEN, B. K. AND SCHUURS, A. H. W. M. 1971. Immunoassay using antigen-enzyme conjugates. *FEBS Letters*, **15**, 232–236.