The Detection of Coxsackievirus RNA in Cardiac Tissue by \textit{in situ} Hybridization

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SUMMARY

A cloned cDNA probe derived from coxsackie B4 virus-infected cell RNA was shown to hybridize to the RNA of a number of different enteroviruses including coxsackie A and B viruses, echoviruses and poliovirus. The probe was used to detect virus-specific RNA sequences in cardiac tissue obtained from patients diagnosed as having a coxsackievirus infection. Virus RNA was detected using the technique of \textit{in situ} hybridization in 46\% (6/13) cases, but none was found in normal, control, cardiac samples. Two distinct patterns of infection were observed. The significance of these differences and the possible uses of the technique are discussed.

INTRODUCTION

\textit{In situ} hybridization as a method for detecting virus-specific nucleic acid within cells has been used for a number of systems, with most attention focused on radioactively labelled probes of the DNA viruses (Haase \textit{et al.}, 1985). We describe here a method for detecting virus-specific RNA sequences using a non-radioactive enzyme detection system based on horseradish peroxidase, similar to the detection systems routinely used in immunocytochemistry.

Coxsackieviruses produce common enteric infections in man and have been shown to be the causative agents of a number of clinically important diseases, particularly in young children (for review, see Melnick, 1984). The major importance of the group B coxsackieviruses has been in relation to the incidence of cardiac dysfunction. It has been known for some time that infection of mice with coxsackie B3 or B4 viruses results in lesions in cardiac tissue. In man, serological studies have indicated a strong link between infection with coxsackie B viruses and the onset of acute pericarditis and myocarditis. Estimates based on studies of antibody titre have suggested that between 25 and 50\% of patients with acute pericarditis have evidence of recent or current infections with coxsackie B viruses and 36 to 65\% of patients with acute myocarditis show a similar relationship (Grist & Bell, 1964; Koontz & Ray, 1971; Schmidt \textit{et al.}, 1973; El-Hagrassy \textit{et al.}, 1980).

Although the evidence is good that coxsackie B virus infection may be associated with acute cardiac disorders leading to fatal arrhythmias, the relationship of virus infection to chronic heart disorders is not so clear. Some studies have suggested a link between coxsackie B virus infection and congestive and hypertrophic cardiomyopathy or otherwise impaired myocardial function (Banatvala, 1983). Recent results using a radiolabelled virus-specific hybridization probe also suggest a link between virus infection and chronic heart disorder (Bowles \textit{et al.}, 1986).

Hallam \textit{et al.} (1986) have reported the use of a hybridization probe for \textit{in situ} hybridization to human biopsy material. We report here the use of this probe to analyse the presence of
coxsackievirus RNA sequences in cardiac tissue taken from patients with an acute coxsackievirus infection using the technique of in situ hybridization.

METHODS

Cells and virus. All of the viruses used, with the exception of coxsackie B4 virus, were clinical isolates prepared in the John Radcliffe Hospital, Oxford. Coxsackie B4 virus was obtained from Dr E. Bell of Ruchill Hospital, Glasgow. Virus stocks were grown and titrated in Vero cells propagated in Eagle's medium containing 10% (v/v) foetal calf serum.

Extraction of cytoplasmic RNA. RNA was extracted from the cytoplasm of infected and uninfected cells according to the method of Kumar & Lindberg (1972) and precipitated by the addition of 2 vol. ethanol followed by overnight storage at -20 °C.

Hybridization to cytoplasmic RNA. Duplicate samples of cytoplasmic RNA were pelleted from ethanol and resuspended in water. The samples were incubated in a boiling water bath for 2 min, chilled, and adjusted to 10 × SSC by the addition of an equal volume of 20 × SSC (SSC is 150 mM-NaCl, 15 mM-sodium citrate). The samples were then applied to a nitrocellulose membrane which had been pre-soaked in 10 × SSC, using a dot-blot manifold (Bethesda Research Laboratories, BRL). After the RNA had been applied, the nitrocellulose was baked for 2 h at 80 °C in a vacuum. The filter was pre-hybridized in a solution of 50% (v/v) formamide, 3 × SSC, 5 × Denhardt's solution (Denhardt, 1966) containing 100 μg/ml of denatured salmon sperm DNA, at 42 °C overnight. Hybridization was performed under the same conditions. Filters were washed extensively at room temperature in 3 × SSC and autoradiographed.

Preparation of coxsackie B4 virus probe. Cytoplasmic RNA was prepared from cells infected with coxsackie B4 virus when they showed extensive cytopathic effect. Single-stranded cDNA was generated using an oligo(dT)12-18 primer and avian myeloblastosis virus reverse transcriptase. Double-stranded cDNA was synthesized as described by Maniatis et al. (1982). Complementary DNA was dC-tailed and annealed to plasmid pBR322 which was dG-tailed at the PstI restriction endonuclease site. Escherichia coli HB101 was transformed with the annealed DNA as described by Maniatis et al. (1982). Colonies containing recombinant plasmids were identified by selecting for resistance to tetracycline and sensitivity to ampicillin. Virus-specific clones were identified by their ability to hybridize to RNA from infected cells, with no hybridization to RNA from uninfected cells. One of the virus-specific clones was designated pB4107 and was shown to contain an insert of approximately 400 base pairs. This was chosen for use as a hybridization probe.

In vitro labelling of probe DNA. DNA was labelled by nick translation, essentially as described by Rigby et al. (1977). For Northern blot analysis probe DNA was labelled using [α-32P]dGTP. For in situ hybridization, probe DNA was labelled using biotin-11-dUTP (BRL) in place of dTTP on the reaction mix; pAT153 DNA was also labelled with biotin-11-dUTP for use as a negative control.

In situ hybridization. Cardiac tissue isolated at post mortem was fixed in 10% formol saline and embedded in paraffin wax prior to sectioning. The paraffin wax was removed by immersing the microscope slide in xylene for 10 min. The tissue samples were then rehydrated by immersion successively in absolute, 90% (v/v), 70% (v/v) and 50% (v/v) ethanol and finally water for 2 min each. Samples were then incubated with proteinase K at 10 μg/ml in water for 5 to 10 min at room temperature to expose the nucleic acid, and washed in water. Endogenous peroxidase activity was removed by incubating the tissue in methanol containing 2% (v/v) hydrogen peroxide for 20 min at room temperature, followed by washing in water.

Hybridization was performed in 42% (v/v) formamide, 3 × SSC containing 50 μg/ml denatured salmon sperm DNA at 37 °C under a sealed coverslip overnight in a humidified atmosphere. Following hybridization, the coverslips were removed and the washed samples successfully in 2 × SSC for 5 min at room temperature, 0.1 × SSC for 10 min at 65 °C and 2 × SSC for 5 min at room temperature. The samples were then immersed in phosphate-buffered saline (PBS) containing 0.1% (v/v) Triton X-100 and washed in PBS at room temperature.

The streptavidin–biotin–horseradish peroxidase detection system (Detek 1-HRP kit; Enzo Biochemicals, New York, U.S.A.) was applied to the samples and incubated for 30 min at 37 °C in a wet chamber. The samples were washed at room temperature in 2 × SSC for 5 min, PBS containing 0.1% (v/v) Triton X-100 for 2 min and washed in PBS.

The substrate solution, 2% (v/v) hydrogen peroxide, 500 μg/ml diaminobenzidine in PBS, was applied to the tissue samples and incubated at room temperature for 10 min. The reaction was stopped by washing with water. The signal was enhanced by treating the samples with 0.1% (w/v) gold chloride for 5 min followed by washing with water. Developer solution was then applied for 90 to 120 s. Developer solution was freshly prepared by mixing equal volumes of 5% (w/v) sodium carbonate and a solution containing 0.2% (w/v) ammonium nitrate, 0.2% (w/v) silver nitrate, 1% (w/v) tungstosilicic acid, 0.185% (v/v) formaldehyde. The reaction was stopped by immersing the samples in 1% (v/v) acetic acid.

The sections were counterstained using a 1% (w/v) solution of Evans’ blue.
In situ hybridization of coxsackievirus RNA

Fig. 1. Dot blot hybridization of $^{32}$P-labelled pB4107 to cytoplasmic RNA from cells infected with coxsackievirus types A1, A7, B1, B2, B4, B6, echovirus types 6, 7 and 11 and poliovirus type 3. Uninfected cell RNA was also included.

RESULTS

Cloning and specificity of hybridization of plasmid pB4107

Cytoplasmic RNA isolated from cells infected with coxsackie B4 virus was cloned into plasmid pBR322 as described. Virus-specific clones were identified by their ability to hybridize with RNA from infected cells and showing no hybridization with RNA from uninfected cells (data not shown). One of these clones, designated pB4107 was analysed in further detail with respect to its ability to hybridize with other enterovirus RNA sequences. Nick-translated, $^{32}$P-labelled pB4107 was hybridized with a nitrocellulose filter containing cytoplasmic RNA isolated from cells infected with a number of enteroviruses. An autoradiograph of the hybridized filter is shown in Fig. 1. It can be seen that pB4107 hybridized with all of the enteroviruses studied under the conditions used. In addition, hybridization was also seen with coxsackieviruses B3 and B5 (data not shown). No hybridization was observed with RNA isolated from uninfected cells. This result showed that pB4107 may be considered a candidate for a general enterovirus probe for detecting virus nucleic acid.

In situ hybridization using pB4107

In view of the possible value of pB4107 as a general enterovirus probe, the plasmid was used as a non-radioactive probe to detect enterovirus sequences in biopsy material. Post-mortem material was obtained from patients diagnosed as suffering from a coxsackievirus infection at the time of death. This diagnosis was based on serology and, when possible, direct isolation of the virus. The tissue was fixed in formol saline prior to preparation for sectioning and had been stored in this form for a considerable time, in one case for 15 years, before this hybridization analysis. As a control, cardiac tissue was also obtained from patients with no evidence of coxsackievirus infection or abnormal cardiac pathology. As an internal control, duplicate
sections of all tissue samples were hybridized with biotin-labelled plasmid pAT153 DNA alone to ensure that all positive hybridization was due to the presence of virus RNA sequences.

A summary of the hybridization results is shown in Table 1. As can be seen, 46% (6/13) of the samples from patients showing evidence of coxsackievirus infection were also shown to be positive for hybridization with pB4107, indicating the presence of virus RNA in the tissue. No hybridization was seen with pAT153 DNA alone. Similarly, no positive hybridization was observed in the control tissue samples when hybridized with either plasmid pB4107 or pAT153 DNA alone. This indicated that the technique of in situ hybridization is capable of specifically detecting virus RNA sequences in fixed tissue. Anomalous hybridization to DNA can be discounted since the DNA in the tissue sections was not denatured and so would not be available for hybridization.

Fig. 2 shows three examples of hybridization, to the samples referred to as 1, 2 and 4 in Table 1; (a), (c) and (e) show the sections following hybridization with control plasmid DNA, and (b), (d) and (f) show hybridization of pB4107 to the next sections in the series obtained from the paraffin-embedded tissues. It can be clearly seen that the hybridization signal, indicated by the
In situ hybridization of coxsackievirus RNA

Table 1. Detection of coxsackievirus RNA sequences in cardiac biopsy material

<table>
<thead>
<tr>
<th>Sample</th>
<th>Coxsackievirus identified*</th>
<th>Clinical symptoms</th>
<th>Hybridization with pB4107</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B4</td>
<td>Myocarditis</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>B2</td>
<td>Myocarditis</td>
<td>Positive</td>
</tr>
<tr>
<td>3</td>
<td>B4</td>
<td>Myocarditis</td>
<td>Positive</td>
</tr>
<tr>
<td>4</td>
<td>B4</td>
<td>Pneumonitis</td>
<td>Positive</td>
</tr>
<tr>
<td>5</td>
<td>B4</td>
<td>Myocarditis</td>
<td>Positive</td>
</tr>
<tr>
<td>6</td>
<td>B4</td>
<td>Myocarditis</td>
<td>Positive</td>
</tr>
<tr>
<td>7</td>
<td>B2</td>
<td>Myocarditis</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>B3</td>
<td>Myocarditis</td>
<td>Negative</td>
</tr>
<tr>
<td>9</td>
<td>B3</td>
<td>Myocarditis</td>
<td>Negative</td>
</tr>
<tr>
<td>10</td>
<td>B4</td>
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<td>B4</td>
<td>Myocarditis</td>
<td>Negative</td>
</tr>
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<td>Negative</td>
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<td>13</td>
<td>B5</td>
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</tr>
<tr>
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<td>Negative</td>
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<td>15</td>
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<td>Normal</td>
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<td>18</td>
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<td>Normal</td>
<td>Negative</td>
</tr>
</tbody>
</table>

* Virus was identified by isolation and serology.

brown pigment which results from the horseradish peroxidase reaction, is very localized within the tissues. In two of the examples shown (a, b and c, f) the adventitia of blood vessels were clearly shown to contain virus-specific RNA as evidence by hybridization to pB4107 but showed no hybridization to the vector DNA sequences. In other tissue sections, for example that shown in (c) and (d) virus RNA can be shown to be present within the cardiac muscle cells themselves. There are several possible explanations for this apparent difference in the pattern of infection as discussed below.

DISCUSSION

Plasmid pB4107, obtained by cloning cytolasmic RNA from cells infected with coxsackie B4 virus, was shown to hybridize to RNA from a number of enteroviruses, both coxsackie A and B viruses, echoviruses and poliovirus, though not to RNA from uninfected cells (Fig. 1). This is similar to the results reported for probes derived from coxsackie B3 virus which were shown to hybridize with RNA from the five other members of the coxsackie B virus group as well as other enteroviruses (Hyypia et al., 1984). The promiscuous hybridization of pB4107 suggested that it would be a good candidate as a general enterovirus-specific, rather than coxsackievirus-specific, probe and may have a possible use as a diagnostic tool.

Using pB4107, hybridization to virus-specific RNA sequences immobilized in situ in cardiac tissue sections was observed in 46% (6/13) of cases where coxsackievirus infection had been implicated by serology or direct virus isolation (Table 1). This confirmed that in these cases, virus had indeed directly infected the heart. The absence of a positive peroxidase reaction when the tissue sections were hybridized with plasmid vector sequences alone confirmed that the positive signal observed when hybridizing with pB4107 was due to the presence of virus-specific sequences.

When studying the tissue sections that gave a positive hybridization signal, it was clear that infections were very localized within the tissue. In addition, two distinct patterns of infection were seen as illustrated in Fig. 2. In one type, the adventitia of one or more blood vessels contained high levels of virus-specific RNA as evidenced by the intensity of the signal. Some of the surrounding cardiac muscle cells also often showed the presence of virus RNA. This type of pattern is seen in Fig. 2(b) and (f). The other pattern of infected cells is shown in Fig. 2(d) in which it can be seen that an area of cardiac muscle cells shows evidence of high levels of virus RNA. The reasons for these two patterns of infected cells are unclear, but there are several
possible explanations. (i) The patterns indicate the route of spread of the virus throughout the body, with the presence of virus RNA in cells surrounding blood vessels demonstrating haematogenous spread of infection whereas the second pattern may indicate that infection of the heart occurred via another route; (ii) the differences in pattern merely indicate different stages in the infection with the virus possibly being introduced via the cardiovascular system and then spreading through the cardiac muscle. If this is the case then the samples in Fig. 2(b) and (f) may represent earlier stages in the infectious process than that seen in (d); (iii) the technique of in situ hybridization is limited by the amount of tissue that can be analysed. Thus, it is possible by looking at sections from several areas of the heart that both patterns would be found and that the differences have no significance in pathogenesis. Use of pB4107 in cases of encephalitis in which coxsackieviruses have been implicated has also been shown that two distinct patterns of infected cells similar to those described above can also be seen (unpublished data).

It is of interest to note that the sites where virus RNA is observed in cardiac tissues using this technique do not correspond to the histological sites of pathological lesions typified by the presence of abnormal cells. This may be because an undetectable level of virus RNA is present in these apparently abnormal cells, or that these aberrant cells do not contain virus and exhibit their abnormal morphology for some other, as yet unknown, reason.

From the results in Table 1, it can be seen that the technique of in situ hybridization is not likely to have general applications as a diagnostic method for coxsackievirus infection. A large proportion of cases where coxsackieviruses were implicated by serology did not give a positive result by hybridization. This may be due to a number of factors. Primarily, this technique allows analysis of only a small amount of material and in a localized infection a positive signal may be missed. This was particularly true of sample 2 shown in Fig. 2(c) and (d). With this sample, sections taken from one region of the embedded tissue were positive as shown, whereas those taken from a sample of another region were negative. Similarly, although sample 10 in Table 1 was negative as indicated, brain sections taken from the same patient were clearly positive. It is possible that if more cardiac sections from patient 10 had been available a positive result would have been obtained.

Another possible problem with this technique which has already been alluded to is that of sensitivity. It has not proved possible to isolate RNA from formalin-fixed tissue in a form in which it can be quantified. Although it is possible to show that as few as 10 copies of a DNA sequence can be detected by in situ hybridization of fixed tissue, no such estimate of sensitivity is possible for RNA sequences (R. Eglin, unpublished data). It is therefore possible that some of the samples that appeared negative in this study did contain virus RNA, but below the limit of detection.

Although in situ hybridization as described here for coxsackieviruses may be limited by the problems described above, hybridization using radiolabelled probes with nucleic acid extracted from unfixed tissue may permit the use of hybridization probes as diagnostic reagents as described by Bowles et al. (1986). However, in situ hybridization of tissue sections can be used to give more detailed information about the pathogenesis of infection, in particular the type and localization of cell infected in the tissue under study. In addition, the technique can be used with any tissue which has been suitably fixed and stored for many years, allowing retrospective studies to be performed in relatively rare or uncommon clinical conditions. In this way we hope to use these probes to study the involvement of coxsackieviruses in a number of clinical disorders.

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REFERENCES


In situ hybridization of coxsackievirus RNA


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