DISCUSSION
In several viral infections, clinical evidences suggesting involvement of heart have been recorded (Abelman, 1973). To obtain proper perspective of influence of viruses in cardiac diseases in South India, virological investigations of 50 such cases were carried out. As already mentioned, serological studies were done against 12 viruses apart from virus isolation attempts in these cases.

For establishing association of viruses to myocardial disease, Grist and Bell (1974) laid down virological categories as follows: Virus isolation and rising antibody titre-I; 4 fold rising or falling antibody titre-II; Antibody titre of >512-III; antibody titre of 128-IV; antibody titre <64-V and virus isolation only-VI and according to them virological categories I-IV carried higher diagnostic significance. According to Lerner and Wilson (1973), following criteria showed the association of a virus to myocardial disease (a) high order association - isolation of virus from heart or pericardial fluid or type specific virus localised in heart by immunofluorescence or antibody labelled with peroxidase or ferritin, (b) moderate-order association - virus isolation from throat or faeces with four-fold antibody rise or concomitant titre of 32 or more of type specific IgM antibody and (c) low order association - virus isolation from throat or faeces, or four-fold
rise in antibody titre or single serum sample titre of 32 or more of IgM antibody. We have tried to make a compromise between criteria for virological categories laid down by Grist and Bell (1974) and that of Lerner and Wilson (1973). The criteria used in this study were (a) Isolation of virus with or without four fold or more rise in antibody titre, (b) a rise of four fold or more in antibody titre, (c) demonstration of a considerably high virus neutralising antibody titre against Coxsackie B1 to B6 viruses, i.e. four times the highest prevalent antibody level in the population, and (d) four fold or more rise in haemagglutination inhibition antibody titres against influenza A and B and mumps viruses and complement-fixing antibody titres against cytomegalovirus.

Twenty nine (58%) among 50 cases investigated in this study satisfy one or other of the above criteria and are considered as virologically "positive". To understand the significance of virus association with cardiac disease, these 29 cases are categorised as given in Table 4. Categories I, II, III and IV were considered as virologically "positive" and carried high diagnostic significance. These 29 cases included 17 myocarditis, 4 pericarditis, 7 rheumatic carditis and a case of cardiomyopathy. On analysis of our data, it is found that 28 cases falling under our categories I (2 cases), II (25 cases) and III (1 case) have a high "threshold of positivity" according to criteria of Grist.
and Bell. According to Lerner and Wilson (1973), Category I (2 cases) belongs to "moderate order association" and Categories II, III and IV belong to "low order association". Thus, the case of pericarditis in which Coxsackie B1 was isolated without serological support (Category IV) was considered as "positive" though Grist and Bell (1974) do not count such cases in their series as "positive".

Categories I and II of our series and that of Grist and Bell (1974) which depend on the four fold rise or fall in antibody titre with or without isolation of virus may be acceptable to different populations in different parts of the world but Categories III and IV of Grist and Bell (1974) i.e. the antibody titres of < 512 and 256 are not applicable elsewhere than in our country. The criteria for the virological "positivity" of antibody titre has to be determined according to the prevalence of Coxsackie B1 to be antibodies in the population where the particular investigation is carried out. Thus, in our investigation, it was found that during the period of this study, an antibody titre of 1:256 was obtained in 83 sera of noncardiac cases & 25 sera of unselected normal persons (Tables-7 to 13). Therefore, to make the criteria for association of Coxsackie B virus to myocardial disease more strict and rigorous, we felt that atleast a titre of 1:1024 (four times the highest titre of 1:256 found that non-cardiac diseases and normal population) should be taken as significant and was labelled as Category III for "positivity". We feel that in a country
where enterovirus infections are common, such a strict application of criteria for association of Coxsackie B viruses to cardiac diseases is necessary. As such, we have considered titres of 512 and less do not carry diagnostic significance in our population. The validity of our virological categories with respect of Coxsackie B virus is also obtained by comparing the results found on investigations on paired serum samples from non-cardiac cases (involving central nervous system and respiratory tract) during the period of present study (Table-6). It can be seen in Table that association of Coxsackie B viruses with cardiac diseases in general is very high except for Coxsackie B6 virus as compared to non-cardiac cases.

During an outbreak of Coxsackie B6 virus infection from October 1974 to March 1975, the virus was even isolated in 24 patients suffering from different non-cardiac diseases such as encephalitis, meningitis and respiratory tract infections and these findings have been reported (Madhavan and Agarwal, 1976). The increase in percentage of "positivity" in non-cardiac cases during this period was due to this outbreak of Coxsackie B6 virus infection. If this period is excluded from our study, the percentage of positivity in non-cardiac diseases is little during the rest of the period. In other words, except for this outbreak, Coxsackie B6 virus infection, in general, has shown little association with non-cardiac diseases. In this study, among 50 cases of cardiac diseases, 19 (38%) were associated with
Coxsackie B viruses and this is comparable to several other reports from different parts of the World (Table-26). It can be seen in Table-26 that higher rates of Coxsackie B virus infection occurs during epidemics. In the present study, most of the cases have occurred sporadically in all seasons of the period of three years of 1973-76. It can also be made out from Table-26 that virus isolation rate from patients in these investigations has been nil or very low except for the reports of Helin et al (1968); Sainani et al (1968) and Sainani et al (1975). Virus isolations had been possible to the extent of 75 percent in the investigations carried out by Helin et al (1968) because all the cases occurred during an epidemic of Coxsackie B5 virus infection. Surprisingly virus isolations have been rather high at 32% and 48% in both the investigations of Sainani et al (1968 and 1975) though they were all sporadic cases. Isolation of viruses in most of the cases of myocarditis/pericarditis has been difficult because cardiac involvement usually does not manifest until 12-15 days after the viral infection and by the time virological investigations are started viraemia and virus excretion in faeces may have ceased. Therefore, in practice, demonstration of four fold or more rise or fall in neutralising antibody titre against the virus, suggesting recent infection, has been taken as evidence of association of virus with the disease process.
<table>
<thead>
<tr>
<th>Clinical group</th>
<th>No. of cases investigated</th>
<th>No. of positive cases</th>
<th>No. of cases with +ve virus isolation (% among positive cases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pericarditis</td>
<td>34</td>
<td>1 (3%)</td>
<td>Nil (0%)</td>
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<td>Myopericarditis</td>
<td>18</td>
<td>16 (89%)</td>
<td>12 (75%)</td>
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<td>57</td>
<td>22 (39%)</td>
<td>7 (32%)</td>
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<tr>
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<td>45</td>
<td>20 (44%)</td>
<td>Nil (0%)</td>
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<tr>
<td>Myopericarditis</td>
<td>153</td>
<td>59 (39%)</td>
<td>4 (7%)</td>
</tr>
<tr>
<td>Myocarditis</td>
<td>18</td>
<td>4 (22%)</td>
<td>Nil (0%)</td>
</tr>
<tr>
<td>Myocarditis/Pericarditis/Myopericarditis</td>
<td>55</td>
<td>19 (35%)</td>
<td>9 (48%)</td>
</tr>
<tr>
<td>Myocarditis/Pericarditis</td>
<td>50</td>
<td>19 (38%)</td>
<td>3 (16%)</td>
</tr>
</tbody>
</table>

Johnson et al (1961)  
Helin et al (1968)  
Sainani et al (1968)  
Koontz and Kay (1971)  
Ayuthya et al (1974)  
Sainani et al (1975)  
Present series
Among the 19 cases of Coxsackie B virus infections, Coxsackie virus B4 accounted for 7 cases, all of which were distributed throughout the span of three years of this study. Among the three cases of Coxsackie B6 virus infection, two occurred during an outbreak of this virus infection (Madhavan et al., 1976). Cardiac diseases associated with this virus type seem to be rare because among 91 cases of this virus infection reported by WHO during 1971-74, only two had cardiac disease (Assaad, 1975).

Except for one case of myocarditis (Case No. 11), all other cases of Coxsackie B virus infection in this study recovered well without any serious complication. Case No. 11, an 18-year-old female with Coxsackie B2 virus infection, developed a chronic pernicious myocarditis as evidenced by protracted and downhill course of illness and died after 18 months of illness and postmortem revealed typical histopathological lesions of myocarditis.

Though the largest group of cardiac illnesses were attributable to Coxsackie B virus infection in this study. Coxsackie A9 and ECHO 9 virus infection have also been found in 1 and 2 cases respectively. Grist and Bell (1968) stress that though group B Coxsackie viruses have been implicated as causing cardiac diseases, reports of Group A Coxsackie virus infection are increasing. In their further investigations of 62 cases, they found that Coxsackie B virus, Coxsackie A virus, ECHO virus and poliovirus infections
occurred in 71%, 16%, 10% and 3% of cases respectively (Bell and Grist, 1972). Enteroviruses are widespread and infection with them are common. Coxsackie B viruses are most commonly associated with these diseases. This may partly be due to the fact that this group of viruses are isolated readily in tissue cultures and the limited number of antigenic types within the group makes it feasible to attempt serological diagnosis. Nothing much is known about Coxsackie A virus infection in cardiac diseases, probably because of the difficulty in the isolation of this group of virus in tissue cultures and there are 24 antigenic types making it difficult to do a serological investigations (Grist and Bell, 1969). Despite the fact, they are widespread and are easily isolated in tissue cultures, ECHO viruses do not appear to be significant in the causation of cardiac disease (Bell and Grist, 1971). In this study, ECHO virus 9 was associated with one case of rheumatic carditis and another of cardiomyopathy. In their analysis of 833 patients from whom ECHO viruses were isolated, Grist and Bell (1971) found virus types 6, 11, 12, 19 and 25 were associated with pleurodynia and/or cardiac disease but ECHO virus 9 did not show significant association with these clinical conditions, though this virus type had been established as etiologically important in cardiac diseases in earlier reports (Monif et al, 1967 and Cherry et al, 1967).

Apart from enteroviruses mentioned earlier, influenza A virus infection was associated with 5 cases (10%) (Case No. 10 and 12, 17, 20 and 37; Table-1) among 50 cases of cardiac
diseases investigated in this study. All the cases occurred in females. There were three cases of myocarditis and two of rheumatic carditis. Both cases of rheumatic carditis (Case No. 10 and 12; Table 1) consistently showed insignificant titres of ASLO ruling out of streptococcal infection. Electrocardiographic changes during influenza A virus infection have been well documented (Gibson et al., 1959; Lewes et al., 1974 and Verel, 1974). Case No. 10, showing 32 fold rise in HI antibody titre against Hong Kong strain of influenza A virus only occurred during early part of 1973 whereas other cases (No. 12, 17, 20 and 37; Table 1) showed more than four fold rise in antibody titres against both A/Hong Kong/1/68 and A/England/42/72 strains showing thereby that Hong Kong strains of influenza A was prevalent even in the early part of 1973. It has already been shown by a serological survey that A/Hong Kong/1/68 strain was prevalent only upto the end of 1971 and was replaced by A/England/42/72 during 1972 (Madhavan and Agarwal, 1976). Therefore, it is likely that the case No. 10 could have been a stray case of influenza A virus infection due to Hong Kong strain.

Cytomegalovirus infection was found in two cases of myocarditis (Case No. 18 and 23; Table 2), but both cases showed highly elevated titres of ASLO, though both of them were not clinically diagnosed as rheumatic carditis nor was there any history of rheumatic fever previously. Careful perusal of literature does not reveal any association of
CAV infection with myocarditis. Serological surveys have shown that this virus infection is fairly common in India (Pal et al., 1972; Madhavan et al., 1974 and Mukundan et al., 1977). Since there was four fold and more rise in C.F. antibody titre against this virus in the two above cases, CAV has been considered to have caused an opportunistic secondary infection, with primary disease process being due to streptococcal infection.

Influenza B virus and mumps virus infections have not been found associated with any case of cardiac disease among 50 cases investigated. It is felt that myocarditis is only rarely associated with these virus infection, as there are only a few case reports available in literature.

Out of a total of 14 cases of rheumatic carditis, 7 (50%) were associated with a virus infection. Burch et al. (1966) have shown development of valvular endocarditis and other changes similar to rheumatic heart disease in experimental infection of mice and monkeys. It is also well-known that nearly one third of patients of late rheumatic heart disease do not give history of rheumatic fever (Sadinani et al., 1968). Five cases (Case No. 4, 9, 10, 12 and 13; Table-1) of rheumatic myocarditis associated with virus infection, in this study, persistently showed low titres of ASLO ruling out streptococcal infection. Two cases of rheumatic carditis both associated with Coxsackie B4 virus infection (Case No. 40 and 48; Table-1) have persistently shown high titres of ASLO and it is likely that superadded
viral infection over streptococcal disease might have occurred in both. It is also of interest to note that even among the seven cases of rheumatic myocarditis not associated with any of the virus infections tested (Table 4 and 5), only two showed evidence of streptococcal disease in the form of high titre of ASLO. For these reasons, it is felt that all cases of rheumatic myocarditis should be investigated for their association with virus infections.

**Cell-mediated immunity in experimental Coxsackie B3 virus myocarditis in guinea pigs:** Experimental viral myocarditis was produced in both adult and young mice by inoculation of Coxsackie B3 virus (Grodums and Dempster, 1959a and 1959b). It was also found that Coxsackie B3 virus strains were particularly cardiotropic for 17 day old and adult mice as compared with other Coxsackie B3 viruses and there was also evidence to show that specific heart lesions developed in adult mice. These pathological changes seem to persist over a prolonged period of time (Grodums and Dempster, 1962). In many of these experiments, the replicating virus has not been isolated from the cardiac tissue after the first week of infection (Pennisone et al, 1973). Therefore, it was felt that immunological responses in the animal may have a role in the limitation of the virus replication and consequently the disease process itself. With this view and taking into consideration the above facts, a study was undertaken to assess the development of cell-mediated
immunity against Coxsackie B3 virus in guineapigs and its role in protection against this virus infection.

The results of experimental Coxsackie B3 virus infection in guineapigs show that, in the course of the virus infection, high cell-mediated immunity develops against the virus as shown by the presence of DH, increase in MML and increased virucidal activity of peritoneal macrophages of both actively and passively immunised animals. CMI lasts in these animals for 3 months and is associated with the presence of live virus in cardiac tissue as has been shown by the isolation of the virus from cardiac tissue monolayer cell cultures upto 12 weeks. It seems likely that the presence of live virus in cardiac tissue is needed for persistence of CMI, since it has been observed that after 16 weeks CMI is not detectable and virus also could not be isolated from cardiac tissue monolayer cell cultures. It is likely that a small dose of (10^2 TCID_{50}) Coxsackie B3 virus results in infection of cardiac tissue producing a latent infection in guineapigs and after a period of about 12 weeks of latent infection, the virus is eliminated. It is difficult to say that heart has a special capacity for sustaining infection for long periods in guineapigs. It is well-known that Herpes simplex produces a latent infection in man in spite of the presence of high quantity of neutralising antibody in the host (Wilson and Miles, 1975b). It is also well-known that viruses can be isolated from tissue cultures made from organs of animals with latent virus infection,
such as SV 40 virus (Sweet and Helleman, 1960) and SV 5 virus (Choppin, 1964) which have been isolated from monkey kidney cell cultures. Therefore, isolation of Coxsackie B3 virus from cardiac tissue monolayer cultures of infected guineapigs in this study is not surprising and could be interpreted as evidence for latent virus infection.

Further, the results of this study also indicate that CMI against Coxsackie B3 virus can be passively transferred to normal guineapigs by transfer of immune spleen cells from actively immunised animals. Such passively immunised guineapigs have been shown to possess CMI against the virus on 5th and 7th day after immune spleen cell transfer as indicated by increase of MFI % and high virucidal activity of peritoneal macrophages specifically stimulated by heat-killed virus antigen. The neutralising-antibody titre against Coxsackie B3 was less than 1:8 in almost all immune spleen cell recipient animals, though it was considerably high in donor guinea-pigs. Therefore, it can be reasonably concluded that only CMI against Coxsackie B3 virus was transferred to recipient animals.

Protective role of CMI against Coxsackie B3 virus has been well brought out by passive immune spleen cell experiments. In such passively immunised animals, as already stated, high CMI was present. These guineapigs when challenged with a large dose (10^5 TCID50) of virus, showed viraemia only during 24 hrs as against its presence up to 5 days in normal animals. It is highly significant.
that the virus could not be isolated even from cardiac tissue monolayer cell cultures of passively immunised guinea-pigs even as early as 7 days after challenge with a high dose of the virus, whereas it could be obtained from cardiac tissue monolayers from immunised animals up to 12 weeks. In addition, subcutaneous inoculation of $10^5$ TCID$_{50}$ dose of Coxsackie B3 virus in 17 normal guinea pigs produced death in 12 (70%) and 5 remaining animals later were sacrificed after 12 weeks (Table 25). All these animals showed histopathological evidence of myocarditis (Fig.6 and 7). On the other hand, all animals passively immunised with immune spleen cell transfer and challenged with identical dose of virus 48 hours later, survived beyond 12 weeks. They also did not show any histological evidence of myocarditis (Fig.8). In experiments of this study any possible effect of neutralising antibodies could be excluded since passively immunised animals showed little or no neutralising antibody. Importance of cell-mediated immunity in resistance to viral infections has well been recognised in man in certain clinical conditions such as measles ( Nahmias et al., 1967), progressive vaccinia (0'Connell et al., 1964; Hanson et al., 1966); in patients under immunosuppressive therapy (Graighead et al., 1967; Schwartz, 1969 and Glasgow, 1970); and in congenital conditions with defective cell-mediated immunity (Fulgeniti et al., 1968). Its importance in experimental viral infections have also been well recognised (Zisman et al., 1969 and Zisman and Allison, 1976).
Mackaness (1970) suggested that ultimate expression of true cellular immunity depends upon the metabolic and functional activity of activated macrophages. It has been shown that macrophages have a role in limitation of the spread of viruses (Goodman and Koprowski, 1962; Allison and Mallucci, 1965; Tompkins et al., 1970). In the present study, high virucidal activity of specifically activated peritoneal macrophages from both actively and passively immunised animals has without doubt been well demonstrated in vitro and it is felt it has a definite role in prevention of establishment of Coxsackie B3 virus in animals passively protected with immune spleen cells from actively immunised donors.

Woodruff and Woodruff (1974), in experimental Coxsackie B3 virus infection of CD-1 and BALB/C mice found that the virus multiplication occurring in cardiac tissue was fully suppressed by 6-8 days after infection. There was also histological evidence of myocarditis in both strains of animals but CD-1 mice survived and most of BALB/C mice died in 8-14 days. Challenge with Coxsackie B3 virus in both strains of mice deprived of T-lymphocytes resulted in suppression of tissue injury of infected hearts of CD-1 and protection of BALB/C mice against lethal infection. They suggested that reaction mediated by T-lymphocytes might be involved in the destruction of myocardial cells. Therefore, they made a study of generation of cytotoxic effect of spleen cells in BALB/C mice.
infected with Coxsackie B3 virus and found the occurrence of highest number of virus specific cytotoxic spleen cells on the 7th day after virus infection (Wong et al., 1977a). They also showed that virus infected neonatal myocardial cells were susceptible to virus specific cytotoxicity of T-lymphocytes and this has an important role in the damage to cardiac tissue (target cells) in Coxsackie B3 virus myocarditis in mice. But it is felt that other factors should also be considered before any definite conclusion is arrived at. Firstly, in Woodruff's experiments, a high dose of \(10^2\) TCID\(_{50}\) was given to mice intraperitoneally and this is a lethal dose for mice particularly for BALB/C mice (though not for CD-1 mice) whereas in the present study only \(10^2\) TCID\(_{50}\) dose was given intradermally to guinea pigs for immunisation. For immunisation especially in experiments involving production of CMI and molecular mediators, only small doses should be used. Secondly, strain difference of two groups of mice seems to be important, since CD-1 mice fully recovered whereas BALB/C mice had high mortality after virus infection and this difference might have been due to qualitative and/or quantitative differences between the virus specific cytotoxicity of T-lymphocytes of these groups of animals, and the same factor may also be applicable in case of guinea-pigs which were used in the present study. If so, the recovery of CD-1 mice might have been due to the development of CMI against Coxsackie B3 virus infection, since no difference was shown in the production of neutralising antibody in both T-lymphocyte deprived and normal animals. It makes
one wonder whether there is a quantitative difference in the amount of cytotoxic factor liberated by sensitised lymphocytes on contact with Coxsackie B3 viral antigen between the two strains of mice. In the present study, experiments were designed to find out the role of CMI in Coxsackie B3 virus infection in guinea-pigs. Protective role of CMI against this virus infection in these animals has been well established by passive immune spleen cell transfer. Such passively immunised guinea-pigs when challenged with $10^5$ TCID$_{50}$ dose of virus showed viraemia only during the first 24 hours as against its presence up to 5 days in normal animals challenged with a similar dose. Moreover, subcutaneous inoculation of $10^5$ TCID$_{50}$ dose of virus produced death in 70% of guinea-pigs but all animals passively immunised with immune spleen cells, challenged with a similar dose survived beyond 90 days. In addition, it has been shown that peritoneal macrophages of both actively and passively immunised guinea-pigs when specifically activated by heat inactivated Coxsackie B3 virus antigen, brought down the virus titre to significantly low levels in 48 hours as compared to that of macrophages from normal animals, indicating their high virucidal activity. It could be possible that this virucidal activity of macrophages, may have a significant role in the final elimination of Coxsackie B3 virus from cardiac tissue even after 16 weeks in immunised animals and it may be an important factor in the prevention of the establishment of the virus in passively immunised guinea-pigs and subsequent determination of the
course of disease. Therefore, it is felt that increased

cell, associated with increased virucidal activity of

macrophages, with considerable reduction in period of

viraemia and increased survival of guinea-pigs after the

virus challenge, has a definite protective role against

E. coxsackie B3 virus myocarditis in guineapigs.