Cellular immunity in congestive cardiomyopathy

The normal cellular immune response

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SUMMARY In vivo and in vitro tests of cellular immunity were studied in patients with congestive cardiomyopathy to determine whether these patients have normal or depressed cell mediated immunity to common environmental antigens and mitogens. No abnormality was found, but this does not exclude the possibility that transient depression of cell mediated mechanisms occurs early in the illness before clinical presentation.

Congestive cardiomyopathy is, by definition, of unknown aetiology¹ and may represent a group of diseases with a common end stage.² The role of viral infection of the heart progressing to cardiomyopathy has received considerable attention,³⁻⁴ and a link with an immunological abnormality has been proposed.⁵⁻⁶ Animal studies suggest that alterations in the immune status at the time of viral infection may influence the type and extent of damage to the myocardium in the short and long term.⁷⁻⁹ Alternatively, once a virus has initiated damage abnormal immunological mechanisms may then perpetuate the damage.¹⁰ Remembering that there may be several different aetiologies in congestive cardiomyopathy, it is also possible that those patients who go on to develop heart disease may have a primary immunological abnormality in contrast to those who recover completely from an acute insult.

The search for a humoral abnormality has not yielded consistent or convincing evidence of antibody mediated damage to the myocardium.¹¹⁻¹² Various abnormalities of cellular immunity have been reported in congestive cardiomyopathy including an abnormal cellular immune status,¹³⁻¹⁴ hypersensitivity and cytotoxicity to cardiac antigen preparations,¹⁴⁻¹⁵ and abnormal suppressor T cell function.¹⁶⁻¹⁸ This study examines the ability of patients with congestive cardiomyopathy to mount a normal cellular immune response compared with normal individuals and patients with ischaemic heart disease.Suppressor and helper T cell numbers were also measured (function was not tested) to see if there was a quantitative abnormality of these cells in cardiomyopathy.

Patients and methods

Three groups of patients were studied (Table 1). The congestive cardiomyopathic group consisted of 27 patients diagnosed according to the classification of Goodwin and Oakley¹: all but two underwent cardiac catheterisation confirming normal coronary arteries and a left ventricular endomyocardial biopsy, the results of which were consistent with congestive cardiomyopathy.¹⁹ The two exceptions had the diagnosis confirmed at necropsy. Twenty eight patients with ischaemic heart disease were matched with the cardiomyopathic group for symptoms using the New York Heart Association classification (Table 1) at the time of first venesection. Eighteen normal healthy controls, age and sex matched with the congestive cardiomyopathic group, comprised the third group.

The general status of patients' cellular immunity was assessed by in vivo skin testing to common antigens, in vitro leucocyte migration inhibition to common environmental antigens, and in vitro lymphocyte transformation to non-specific mitogens. Suppressor and helper T cells were counted in eight subjects from each of the three groups using mouse monoclonal antibodies produced against T cells.

The reagents used were: tuberculin PPD (Evans Medical Limited); streptokinase/streptodornase (Lederle Laboratories); Candida albicans (Bencard); physiological saline; BCG vaccine (Evans Medical Limited); preservative free heparin sodium (1000
Table 1  Details of patient groups

<table>
<thead>
<tr>
<th>Group</th>
<th>No of patients</th>
<th>Sex (M/F)</th>
<th>Age (yr) (mean (SD))</th>
<th>NYHA class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congestive cardiomyopathy</td>
<td>27</td>
<td>23/4</td>
<td>50 (11)</td>
<td>I 7 7</td>
</tr>
<tr>
<td>Ischaemic heart disease</td>
<td>28</td>
<td>23/5</td>
<td>56 (8)</td>
<td>IIa 6 6 10 3</td>
</tr>
<tr>
<td>Normal</td>
<td>18</td>
<td>15/3</td>
<td>48 (12)</td>
<td>III 6 3</td>
</tr>
</tbody>
</table>

NYHA, New York Heart Association classification.

LEUCOCYTE MIGRATION INHIBITION

The antigens used were BCG vaccine and streptokinase/streptodornase. The optimal antigen concentrations were determined before the study to avoid antigen toxicity, which hinders cell migration. A viability count was used to assess cytotoxicity to human leucocytes; >15% cell death and clumping of cells was taken to represent cytotoxicity. A dose-response curve was constructed and antigen concentrations below the cytotoxic range were used in the studies. For each test, a BCG pellet was suspended in 1 ml water for injection and varying concentrations made using tissue culture medium (RPMI with 10% fetal calf serum). The streptokinase/streptodornase was prepared as for the skin test and three different concentrations made at the time of the test using a tissue culture medium. A fresh sample of venous blood (16–20 ml) was collected from each patient in a sterile container with 0-4 ml heparin. Leucocytes were separated by adding 10 ml dextran and incubating at 37°C for 30 minutes. The leucocyte rich layer was removed and centrifuged at 400 g for 10 minutes at room temperature. The supernatant was discarded and the cells resuspended and washed in medium three times. The cells were then counted and resuspended at a dilution of 30 x 10⁶ cells/ml. The cells were packed in capillary tubes (20 µl Volutopette Pipets, Dade), plated at one end with hard wax, and centrifuged at 300 g for 10 minutes. The antigens were put into the wells of a leucocyte migration plate (Sterilin) in a random manner, three wells for each antigen concentration and three wells containing tissue culture medium alone to act as a control. The capillary tubes were scored at the cell-fluid interface and two capillaries placed in each well secured by a plug of soft wax; the wells were covered with a coverslip, and an airtight seal ensured by using soft wax on the rim of the wells. Each plate was incubated for 18 hours at 37°C. The plates were then projected and the areas of migrating cells drawn on to paper; the areas were measured using a planimeter and the mean value of each antigen dilution divided by the mean control value (migration index). The coefficient of variation for the control wells had to be <10%. A migration index <0-80 represented inhibition for both BCG and streptokinase/streptodornase.

LYMPHOCYTE TRANSFORMATION

Phytohaemagglutinin and pokeweed mitogen were the mitogens used. Fresh venous blood was obtained from each patient in a sterile container with 0-4 ml heparin. The blood was layered on to Ficoll-Triosil and centrifuged at 400 g for 30 mins. The lymphocytes were removed from the serum-Ficoll-Triosil interface and washed in RPMI at 400 g for 10 minutes. Washing was repeated three times. The lymphocytes were counted and diluted to a concentration of 1 x 10⁶ cells/ml in medium (10% AB serum in RPMI). Then a 0-1 ml cell suspension was placed in each well of a microtitre plate (Sterilin). A range of concentrations for both antigens was prepared using RPMI and each concentration added to three wells; each plate had triplicate control wells containing no antigen. The plates were incubated at 37°C in a humidified gassed incubator for 72 hours for phytohaemagglutinin and for seven days for pokeweed mitogen. Twenty four hours before the end of incubation, 0-5 µCi (0-02 MBq) tritiated

units/ml) (Duncan, Flockhart and Co Ltd); RPMI medium 1640 (Gibco Biocult) supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin; fetal calf serum (Gibco); AB serum (Blood Transfusion Service); dextran 110 in physiological sodium chloride (Fisons); Ficoll-Triosil (Pharmacia, Nyegaard); tritiated thymidine (Amersham); scintillation fluid; monoclonal antibody OKT3, OKT4, OKT8 (Orthoclone); fluorescein conjugated antimouse IgG (Meloy); and glycerol with paraphenylenediamine (PPD).

Intradermal skin tests were performed using 5 units tuberculin PPD, 10-2-5 units streptokinase/streptodornase, and 100 units Candida albicans. Each vial of streptokinase/streptodornase was reconstituted in saline, dialysed in saline for 48 hours to remove the preservative thiomersal, and further diluted to 100 units/ml streptokinase with saline. The skin tests were performed after the in vivo studies were complete and the response of the skin tests measured and graded at 48 hours: grade 1 represented erythema ≥10 mm without induration or with induration up to 5 mm; grade 2, induration between 6 and 10 mm; grade 3, induration between 11 and 20 mm; grade 4, induration >20 mm.21
thymidine was added to each well. After incubation, the cultures were harvested using a multiple cell culture harvester (Skatron, Flow). The dried harvester filters were placed in vials with 2 ml scintillation fluid and the degree of incorporation of label into cellular DNA measured using a scintillation counter. The results were expressed as the ratio of disintegrations per minute for each antigen dose to the unstimulated control value after correction for quenching and background subtraction.20

**SUPPRESSOR AND HELPER T CELL MEASUREMENTS**

Lymphocytes were prepared as for lymphocyte transformation. After counting, the lymphocytes were diluted to a concentration of $5 \times 10^6$ lymphocytes/ml with RPMI medium. Two hundred microlitres (1 x $10^6$ cells) were put into four glass tubes and the monoclonal antibodies added: 5 $\mu$l of monoclonal antibody to helper cells (OKT4), to suppressor cells (OKT8), and to all mature peripheral T lymphocytes (OKT3) were each added to one glass tube, and the fourth had no monoclonal antibody in order to act as a control. The tubes were kept in an ice bath and mixing allowed to take place for at least one hour at 4°C. The cells were washed three times with 5% fetal calf serum in RPMI medium for 10 minutes at 700 g at 4°C. One drop (0.03 ml) fluorescein conjugated anti-mouse IgG was added to each test tube including the control and the cells allowed to stand for half an hour in an ice bath at 4°C. The cells were washed three times at 4°C with 5% fetal calf serum in medium as before and the supernatant discarded. The cells were resuspended gently, mounted in glycerol PPD, and viewed under the fluorescence microscope. Three fields containing 30-40 lymphocytes each were counted and the number showing fluorescence expressed as a percentage of the total lymphocytes counted.

**MICROSCOPY**

A Leitz Ortholux II microscope with Epi illumination using a super pressure mercury lamp source (HBO 50), a Balzer filter exciter, a red cutoff filter (RG8), and a yellow barrier filter (Kodak Wratten) was used.

**STATISTICAL ANALYSIS**

Comparisons between groups for tuberculin and streptokinase-streptodornase skin tests were made using the $x^2$ test for independent samples or the Fisher exact probability test where sample numbers were small.24 The Kruskal-Wallis one way analysis of variance was used to test statistical significance of transformation and migration inhibition results.24

**Results**

Table 2 shows the results of the skin tests. There were no significant differences in the number of positive responders between the groups using tuberculin ($0.5 > p > 0.3$), streptokinase ($0.7 > p > 0.5$), or candida. Using tuberculin, 58% of the congestive cardiomyopathy group and 57% of the normal group had a grade 2 or higher positive skin test compared with only 25% of the ischaemic heart disease group; similarly, 44% of congestive cardiomyopathic patients and 46% of the normal group had at least a grade 2 response with streptokinase compared with only 19% in the ischaemic group. Four patients in the cardiomyopathy group and six in the ischaemic heart disease group did not give a response with any antigen; this was significant for the ischaemic heart disease group when compared with the normal controls ($p = 0.01$) but not for the congestive cardiomyopathy group.

Figure 1 shows the leucocyte migration inhibition results at the highest concentration of each antigen. Seventeen of 27 cardiomyopathy patients, 20 of 27 ischaemic patients, and 11 of 18 normal controls were inhibited by BCG antigen at its highest concentration. Similarly, eight, nine, and six of the cardiomyopathic, ischaemic, and normal groups respectively were inhibited with streptokinase/streptodornase antigen.

<table>
<thead>
<tr>
<th>Antigen and patient group</th>
<th>Grade of response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Tuberculin PPD:</td>
<td></td>
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<tr>
<td>Congestive cardiomyopathy</td>
<td></td>
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<tr>
<td>Ischaemic heart disease</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Streptokinase/streptodornase:</td>
<td></td>
</tr>
<tr>
<td>Congestive cardiomyopathy</td>
<td></td>
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<tr>
<td>Ischaemic heart disease</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Candida albicans:</td>
<td></td>
</tr>
<tr>
<td>Congestive cardiomyopathy</td>
<td></td>
</tr>
<tr>
<td>Ischaemic heart disease</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td></td>
</tr>
</tbody>
</table>
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1.4-1.3-1.2-1.1-1.0-0.9-0.8-0.7-0.6-0.4-0.3-0.2-0.1

Table 3 Mean (SD) percentage of helper and suppressor T cells

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Helper</th>
<th>Suppressor</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congestive cardiomyopathy</td>
<td>45 (8)</td>
<td>18 (8)</td>
<td>52 (10)</td>
</tr>
<tr>
<td>Ischaemic heart disease</td>
<td>43 (9)</td>
<td>15 (8)</td>
<td>57 (14)</td>
</tr>
<tr>
<td>Normal</td>
<td>40 (9)</td>
<td>23 (5)</td>
<td>58 (11)</td>
</tr>
</tbody>
</table>

stimulation with the mitogens, although the range of response was very wide in each group. None of the differences between the groups was statistically significant.

Table 3 shows the mean percentage value and standard deviation for the helper, suppressor, and total mature T lymphocytes in each group. The percentage of control staining was <2%. There were no group differences.

Discussion

Leucocyte migration inhibition, delayed hypersensitivity skin tests, and lymphocyte transformation are primarily tests of cell mediated immune responses and involve the release of soluble factors—lymphokines—which are not antibodies.

Lymphokines are released from lymphocytes previously sensitised to the antigen under examination in the migration inhibition and skin tests. BCG, tuberculin PPD, Candida albicans, and streptokinase represent common environmental antigens to which many people have had previous exposure, and therefore leucocyte migration inhibition and positive skin

Fig. 1 Migration index using (a) BCG antigen (30 μg/ml) and (b) streptokinase (SK)/streptodornase (3000 u/ml SK) for each subject in the three groups. Values <0.8 represent inhibition. The mean value and standard error for each group is indicated. CCM, congestive cardiomyopathy; IHD, ischaemic heart disease.

Lower antigen concentrations showed less inhibition in all groups, the mean migration indices tending to approach 1.00.

Figure 2 shows the lymphocyte transformation results for the three highest concentrations for each antigen. All patients and normal controls showed

Fig. 2 Lymphocyte transformation to (a) phytohaemagglutinin (PHA) and (b) pokeweed mitogen (PWM) expressed as the mean ratio of disintegrations per minute for each antigen concentration to the unstimulated control for each patient group. CCM, congestive cardiomyopathy; IHD, ischaemic heart disease; SEM, standard error of the mean.
tests would be expected to occur in a proportion of a normal population.25–28,29

Both T and B lymphocytes can be stimulated by antigen to form blast cells and synthesise DNA; the incorporation of tritiated thymidine during this process allows the blastogenic activity to be measured.10 Phytohaemagglutinin and pokeweed mitogen are non-specific stimulants of lymphocytes not requiring presensitisation31,32 and stimulate T cells mainly, B cells predominantly only secondarily.21,33

These tests are useful in determining whether cellular immunity is normal or depressed, but they are testing only a small part of a complex system.27

In our study patients with congestive cardiomyopathy did not differ in any way from the normal control group when tested by leucocyte migration, lymphocyte transformation, or skin testing; this contrasts with other studies. Das et al claimed that over 40% of patients with congestive cardiomyopathy had abnormal cell mediated immunity.13 In fact, their results for leucocyte migration inhibition showed no significant mean group difference, but 42% of patients with cardiomyopathy lay outside the 90% confidence limits of the control group. Lymphocyte transformation results were not significantly different from those of a miscellaneous heart group, but both were significantly different from the normal control group, suggesting that the abnormality was not specific to cardiomyopathy. No other studies have undertaken a comprehensive survey of cell mediated immunity;34 the results of Das et al suggest that only single indices of cell mediated immunity were tested in each group, and there were no observations that abnormal lymphocyte transformation results, for instance, coincided with the same population of congestive cardiomyopathy patients showing abnormal leucocyte migration inhibition.

The ischaemic heart disease group had similar migration and transformation results but responded poorly to skin testing when compared with both the cardiomyopathic and normal groups. A positive skin test in this study was defined according to Bates et al,21 but not everyone would accept erythema alone, or induration <5 mm, as positive.35 In this case, the discrepancy between the ischaemic group and the cardiomyopathy and normal groups is emphasised. Depressed cellular responsiveness can occur with increasing age, after viral illness or general anaesthesia, and where there is severe debilitating disease; leucocytosis, fever, anaemia, and weight loss are also strongly interrelated.36 Few of these factors apply to ischaemic heart disease and those which might also apply to congestive cardiomyopathy (except for the higher mean age of the ischaemic group). It was not possible to differentiate the groups by severity of symptoms or number of deaths (seven in the cardiomyopathy group, six in the ischaemic heart disease group) during the study. No quantitative abnormality of suppressor or helper T cells was found, but function was not assessed.

Patients with congestive cardiomyopathy appear to have normal cellular immunity by the time they are diagnosed. This does not exclude the possibility that cellular immunity may be depressed transiently at the time of the initiation of the disease, for example after a viral illness, but it does suggest that patients with congestive cardiomyopathy have the potential to mount a type IV hypersensitivity response which may be important in the pathogenesis of the disease.

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