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Cell-mediated immunity to liver antigen in toxic liver injury

II. ROLE IN PATHOGENESIS OF LIVER DAMAGE

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SUMMARY

The possible pathogenetic role of lymphocytes sensitized to liver antigens was investigated in CBA mice in which sublethal hepatic necrosis had been induced by carbon tetrachloride (CCl_4). Sensitized lymphocytes from CCl_4-treated mice were administered to syngeneic recipients. The recipients developed sensitivity to liver antigens but showed no evidence of liver damage. The cell mediating the immune response both in the donor and the recipient was a T cell. This was demonstrated further by studies involving mice rendered T cell deficient. These mice did not develop sensitized lymphocytes when they were treated with CCl_4 but the extent of liver damage was similar in both T cell-depleted and intact animals. These findings suggest that T cell sensitization to liver antigens occurs as a result of toxic liver damage and does not play a role in the pathogenesis of the hepatic necrosis.

INTRODUCTION

Although lymphocytes sensitized to liver antigens have been demonstrated in a variety of acute and chronic liver diseases (reviewed by Smith, Cooksley & Powell, 1977) the role that these cells play in the pathogenesis of the liver damage is unclear. All of the disorders in which these sensitized cells are present are associated with hepatic necrosis and it is therefore possible that the sensitization is an effect of the necrosis rather than a cause.

There have been a number of reports in which it has been suggested that sensitized lymphocytes may be involved in the pathogenesis of experimental liver damage (Warnatz *et al.*, 1967; Scheiffarth, Warnatz & Mayer, 1967; Scheiffarth, Schmidt & Warnatz, 1967; Warnatz, Scheiffarth & Leibelt, 1968; Warnatz, Scheiffarth & Schmeissner, 1975; Maclaurin, 1971). These studies involved transfer of lymphocytes from experimentally liver damaged animals to syngeneic recipients resulting in liver pathology in the recipients. In some of these reports T cells were shown to be the cell responsible for the observed responses (Warnatz, Scheiffarth & Schmeissner, 1975).

The present study was undertaken to investigate further the relationship of cause and effect of experimental toxic liver damage and lymphocytes to liver antigens. This was undertaken in two ways; firstly, the effect of transferring sensitized lymphocytes to syngeneic recipients was investigated and, secondly, the effect of hepatotoxin administered to mice who were unable to mount a T cell response was observed.

A further aim of this study was to investigate the role of T cells in the immune responses observed. This was accomplished by experiments in which the sensitized cells were depleted or enriched with T cells prior to transfer and by studying T cell-depleted mice.

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MATERIALS AND METHODS

The animals used, the experimental procedure for the induction of sublethal hepatic necrosis and the preparation of liver and spleen antigens were as described in the accompanying paper (Smith, Cooksley & Powell, 1980).

Transfer of unseparated cells. Inbred mice of the CBA strain were treated with intraperitoneal CCl_4 (0·1 ml/kg) in 0·2 ml paraffin. Control mice were given paraffin alone. All animals were killed 72 hr after injection.

Suspensions of pooled spleen and lymph node cells were obtained from both CCl_4 treated and control mice and assayed for the presence of sensitivity to a liver antigen preparation by lymphocyte transformation and macrophage migration inhibition as described in the accompanying paper (Smith *et al.* 1980). The response of these cells to PHA was also assessed.

Aliquots of each of these cell suspensions were administered intravenously to syngeneic recipients. Each recipient received 50×10^6 washed viable cells in 0·2 ml medium.

The recipients were killed at various times after the cell transfer. Spleen cells from the recipients were examined for sensitivity to a liver antigen preparation (using the same methods as for the donor cells) and in some cases to a spleen antigen preparation. Histological assessment of liver tissue from recipients was also undertaken. Serum aspartate amino transferase was measured using the same method as described previously (Smith *et al.* 1980).

Preparation of T lymphocyte-depleted cell suspensions. Spleen cell suspensions were made from both CCl_4-treated and control mice as described above. These suspensions were treated with anti-θ serum and complement to remove T cells. The serum was prepared from AKR mice who had received intraperitoneal immunization for 9 weeks with CBA thymus cells by the method of Reif & Allen (1964).

Anti-θ serum (0·5 ml) which had been heat inactivated at 56°C for 30 min prior to use was added to each aliquot of 100×10^6 cells and this mixture incubated at 37°C for 30 min. The cells were then washed twice and exposed to complement. The source of the complement was a 1 in 6 dilution of guinea-pig serum which had been absorbed with agarose. The diluent used was RPMI 1640. The agarose absorption was carried out first by diluting the guinea-pig serum 1 in 3 and adding 80 mg of agarose per ml of undiluted serum. The serum–agarose mixture was placed on ice for 1 hr and mixed frequently during that time. Following this the mixture was centrifuged and the supernatant taken and further diluted to give a final dilution of 1 in 6. To each aliquot of 100×10^6 cells was added 2 ml of the 1 in 6 guinea-pig serum. The cells were incubated with complement for 30 min at 37°C and then washed and viable cells counted.

Preparation of T lymphocyte-enriched cell suspensions. The method used to isolate this fraction of cells involved the passage of the spleen cell suspension through a nylon wool ('Leukopak') column and removal of adherent cells (most of which are non-T cells) as described by Julius Simpson & Herzenberg (1973).

Transfer of separated cells. Aliquots of both T-enriched and T-depleted cell suspensions were tested for the presence of cells sensitized to liver antigens and response to PHA. Further aliquots of these suspensions were administered intravenously to syngeneic mice. Each recipient received 50×10^6 viable cells in 0·2 ml medium. All recipients of cells were killed after 72 hr and studied in the same way as the recipients of the unseparated cells (described above) except that lymphocyte transformation testing was omitted.

Studies of T lymphocyte-depleted mice. CBA mice were thymectomized at the age of 4–6 weeks by the method of Miller (1960). Sham operations were performed on a similar group of mice to act as controls. The sham procedure involved exposure of the thymus but not its removal.

The surviving animals (both thymectomized and sham-operated) were maintained on a normal pellet diet in routine animal house conditions. Six weeks after the operative procedure the mice were subjected to lethal whole body irradiation. The radiation was applied using a Siemens Stabilian orthovoltage radiotherapy unit which was adjusted to supply a dose rate of 130 rads/min. Both sides of the animal were irradiated and a total dose of 750 rads (calculated at the midline of the animal) was delivered to each animal. Whilst being irradiated the animals were held in perspex boxes which were small enough to prevent the mice turning around. This manoeuvre ensured a uniform dose to each animal.

Each irradiated animal was administered (via a tail vein) with $5{\cdot}0 \times 10^6$ fresh, viable bone marrow cells from syngeneic CBA mice. This strain has virtually no T cells or T cell precursors in the bone marrow (Basten, personal communication). The cells were administered within 1 hr of irradiation.

Eight weeks later these mice (both the thymectomized and the sham-operated groups) were administered either CCl_4 (0·1 ml/kg) in 0·2 ml paraffin intraperitoneally or 0·2 ml paraffin alone. Seventy-two hours later the animals were studied as described above. This involved obtaining serum for AST determination, liver tissue for histological assessment and spleen cells for determining lymphocyte responses to PHA and liver antigen.

RESULTS

The values which signify lymphocyte sensitization for the tests of lymphocyte function have been described in the accompanying paper (Smith, *et al.*, 1980).

Transfer of unseparated cell suspensions

(a) *Donors.* Pooled spleen cells from the control mice did *not* react to liver antigen in the lymphocyte

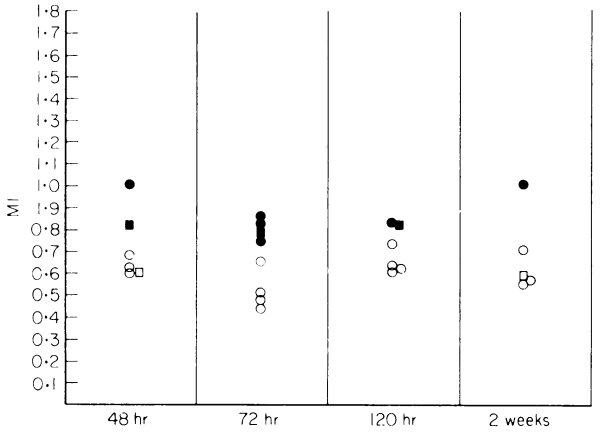

FIG. 1. Migration indices obtained from lymphocytes of recipients of sensitized cells studied at various times after administration. Circles represent recipients of spleen cells and squares recipients of lymph node cells. Open symbols represent significant migration inhibition.

transformation test (geometric mean increase in tritiated thymidine uptake was −371 d.p.m.) or in the macrophage migration inhibition assay (migration index was 0·91) but had a normal response to PHA.

Pooled spleen cells from the animals treated with CCl_4 reacted to liver antigen in the lymphocyte transformation test (geometric mean increase in tritiated thymidine uptake was 3129 d.p.m.) and in the macrophage migration inhibition assay (migration index was 0·57). A normal response to PHA was also observed with these cells.

(b) *Recipients.* Liver histology was normal in recipients of all cells as was serum AST (recipients of cells from control animals, 56 ± 26 s.d., $n = 12$; recipients of cells from CCl_4-treated animals, 57 ± 30, $n = 25$). All these animals also had a normal response to PHA.

Sensitized lymphocytes were not detected in the recipients of any of the cells using a lymphocyte transformation test. This was true even though the donor cells were sensitive to liver antigen in both assays of cell-mediated immunity used.

When macrophage migration inhibition was used to measure cell-mediated immunity sensitized cells could be detected in the recipients of sensitized cells from CCl_4-treated donors (Fig. 1). The sensitization was detectable for up to 2 weeks after cell transfer. None of the recipients of cells from control mice demonstrated this reaction.

Aliquots of four of the spleen cell cultures from recipients which produced a positive migration inhibition test with liver antigen were tested with spleen antigen and no sensitization to spleen antigen was observed. The migration indices were 0·79, 0·83, 0·81 and 0·91.

Transfer of T lymphocyte-depleted cell suspensions

(a) *Donors.* Aliquots of the pooled spleen cells from CCl_4-treated animals reacted to liver antigen in the macrophage migration inhibition assay and in the lymphocyte transformation test. The migration index was 0·69 in the former and in the lymphocyte transformation test the geometric mean increase in tritiated thymidine uptake was 2590 d.p.m. The pooled cells from control animals were not positive in either assay. The migration index was 0·83 and the geometric mean increase in tritiated thymidine uptake was 1066 d.p.m. respectively. PHA response of both cell suspensions was normal. The geometric mean uptake of tritiated thymidine uptake of triplicate cultures was 156, 243 and 210, 109 d.p.m. respectively.

Aliquots of cells from each of these two suspensions which were treated with anti-*θ* serum were tested in both the lymphocyte transformation test and macrophage migration inhibition assay with liver antigen. Neither of the cell suspensions demonstrated detectable response to liver antigen in either assay. The migration indices were 0·91 and 0·71, and geometric mean increases in tritiated thymidine uptake of triplicate cultures were 134 and −93 d.p.m. respectively. Furthermore, the responses of both cell

TABLE 1. AST values in recipients of subpopulations of cells

Populations of cells administered	AST values (Karmen units)*
Non-sensitized cells	70±18 (n = 6)
Sensitized cells	81±33 (n = 10)
Anti-θ serum-treated non-sensitized cells	63±17 (n = 3)
Anti-θ serum-treated sensitized cells	58±29 (n = 2)
T enriched non-sensitized cells	76±36 (n = 2)
T enriched sensitized cells	85±54 (n = 3)

* Mean±s.d.

suspensions to PHA were markedly depressed. The geometric mean of tritiated thymidine uptake of triplicate cultures were 2992 and 3076 d.p.m. respectively.

(b) *Recipients.* Liver biopsies were examined in all recipients of the cells and were all normal. Similarly, AST values were all within the normal range (Table 1).

All the recipients of cells demonstrated normal PHA-induced responses.

Macrophage migration inhibition results demonstrated significant inhibition in three out of five

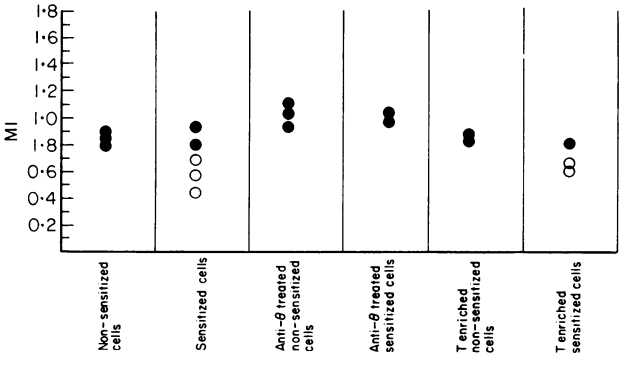

FIG. 2. Migration indices in response to liver antigen produced by lymphocytes from recipients of different cell suspensions. Studied at 72 hr after injection of cells. Open symbols represent significant migration inhibition.

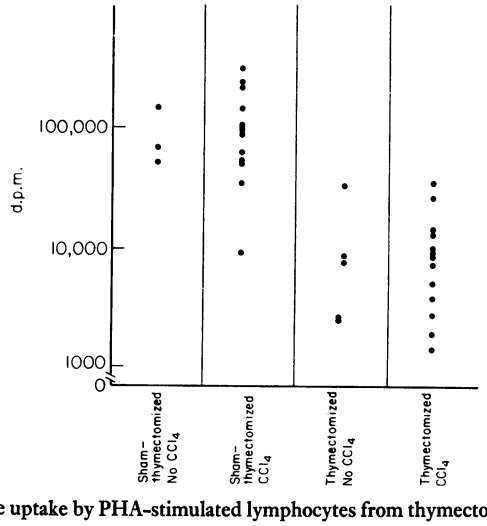

FIG. 3. Tritiated thymidine uptake by PHA-stimulated lymphocytes from thymectomized and sham-thymectomized control and carbon tetrachloride treated mice. Each point represents the geometric mean of triplicate cultures from individual animals.

recipients of sensitized cells (from CCl_4-treated donors) but not in the recipients of the other three groups of cells (Fig. 2).

Transfer of T lymphocyte-enriched cell suspension

(a) *Donors.* Pooled spleen cells from a further group of CCl_4-treated donors reacted to liver antigen in both the macrophage migration inhibition test (migration index was 0·66) and in the lymphocyte transformation test (the geometric mean increase in tritiated thymidine uptake was 2181 d.p.m.). Cells from paraffin-treated animals were negative in both assays (the migration index was 0.78 and the geometric mean increase in tritiated thymidine uptake was 567 d.p.m.). PHA responses of both cell suspensions were normal.

(b) *Recipients.* Liver biopsies and serum AST values were normal in all the recipients studied (Table 1) and the AST values did not differ significantly from those seen in recipients of other cell suspensions or normals. PHA response was normal in lymphocyte cultures from all the recipients of cells studied. Macrophage migration inhibition demonstrated significant inhibition in five of the recipients of sensitized cells both untreated cell suspensions and T lymphocyte-enriched cell suspensions (Fig. 2).

T lymphocyte-depleted animals

(a) *Assessment of effectiveness of operation.* Thymic tissue was present macroscopically in all the sham-operated animals but was absent in 85% of thymectomized animals. The 15% with residual thymic tissue occurred more commonly in those mice operated on early in the course of these studies. Those thymectomized mice with residual thymic tissue were not studied further.

(b) *Assessment of liver damage.* Serum AST values of the animals given paraffin alone were within the normal range and there was no significant difference ($P>0·05$ using analysis of variance) between the values in the thymectomized and sham-operated animals (Table 2).

The levels of serum AST were elevated in all the animals administered CCl_4 but there was no significant difference ($P>0·05$, using analysis of variance) between the values in the thymectomized, sham-thymectomized and unoperated mice who had been administered CCl_4 72 hr prior to testing (Table 2).

Liver biopsy appearances were normal in all the animals administered paraffin alone, both thymectomized and sham-operated. The histological appearances of the liver biopsies in all animals given CCl_4 were abnormal and demonstrated a similar degree of resolving centrizonal necrosis as was seen in the livers of unoperated animals 72 hr after the administration of CCl_4.

(c) *PHA responses.* The response of the lymphocytes from the various animals to PHA is depicted in Fig. 3. All of the thymectomized animals demonstrated an abnormally reduced uptake of tritiated thymidine in response to PHA. This applied to both those thymectomized animals who had received CCl_4 and those who had received paraffin alone. There was no significant difference between these two groups of thymectomized mice.

The responses of the cells from the sham-thymectomized animals to PHA were all within a previously established range (C. Smith, unpublished data) with the exception of two cultures from animals treated

TABLE 2. AST values in T lymphocyte-depleted mice and in sham-operated controls (compared with normal mice). Mean ± s.d. (Karmen units)

	Sham-thymectomized	Thymectomized	Untreated*	
No CCl_4	39 ± 5 ($n = 4$)	49 ± 13 ($n = 4$)	64 ± 23 ($n = 46$)	$P>0·05$
72 hr after CCl_4 treatment	257 ± 102 ($n = 9$)	322 ± 240 ($n = 8$)	296 ± 284 ($n = 8$)	$P>0·05$

* Data from Smith *et al.* (1980)

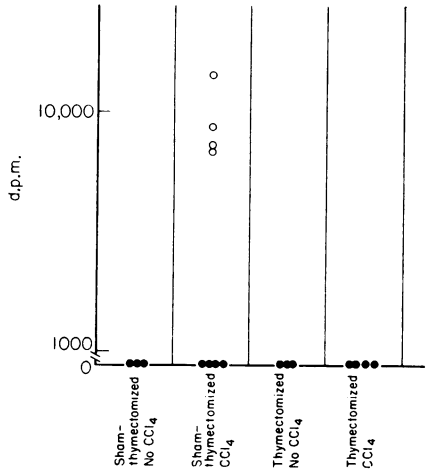

FIG. 4. Tritiated thymidine uptake by lymphocytes from thymectomized and sham-thymectomized control and carbon tetrachloride-treated mice. Open symbols represent significant stimulation of tritiated thymidine uptake.

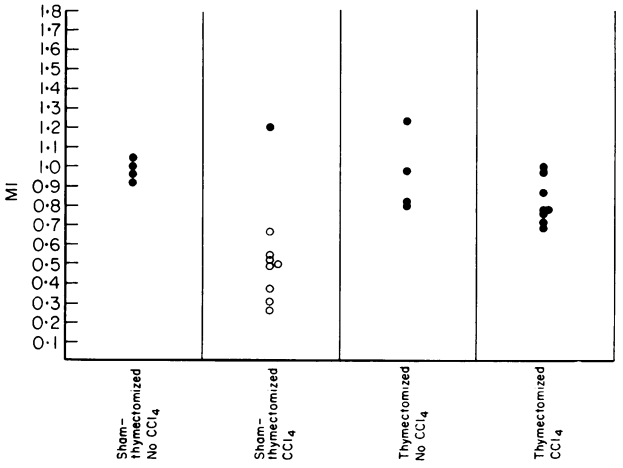

FIG. 5. Migration indices produced by cultures of lymphocytes from thymectomized and sham-thymectomized control and carbon tetrachloride-treated mice. Open symbols represent significant inhibition of migration.

with CCl_4. Aliquots of these cell suspensions subsequently proved not to respond to liver antigen but these results were not taken into account in further analyses of the data as the depressed non-specific lymphocyte function may possibly have resulted in a false negative response to a specific antigen.

(d) *Lymphocyte transformation tests.* The only group of cultures which demonstrated significant increase in tritiated thymidine uptake in the presence of liver antigen were from the sham-thymectomized animals treated with CCl_4 (four out of eight positive). None of the cultures from the thymectomized animals, whether treated with CCl_4 or not, was positive (Fig. 4).

(e) *Macrophage migration inhibition.* Similar results were obtained with the macrophage migration inhibition assay. Eight out of nine cultures from sham-thymectomized animals treated with CCl_4 produced significant migration inhibition in the presence of liver antigen. None of the other three groups was positive (Fig. 5).

DISCUSSION

These results have demonstrated that the transfer of lymphocytes sensitized to liver antigens to syngeneic

recipients did not result in any detectable changes in liver histology or AST levels. The sensitized lymphocytes from the recipient demonstrated a similar specificity to that observed with lymphocytes from CCl_4-treated mice (Smith *et al.*, 1980). The transferred sensitivity was observed only when the macrophage migration inhibition test was used but not with the lymphocyte transformation test despite both assays being positive when donor lymphocytes were tested. This finding may merely reflect a greater sensitivity of the macrophage migration inhibition test in this system.

Scheiffarth, Warnatz & Mayer (1967) and Warnatz *et al.* (1967) produced experimental liver lesions in mice using multiple doses of CCl_4 and were able to transfer this lesion to syngeneic animals by means of lymphocytes from the liver-damaged mice. They were also able to demonstrate that tritium-labelled lymphocytes from such CCl_4-treated animals accumulated in the livers of the recipients to a greater extent than the lymphocytes from control mice. In these studies, however, the liver histology in the recipients of cells from CCl_4-treated mice showed only mild Kupffer cell hyperplasia and periportal cell infiltration but no significant hepatocellular damage. The CCl_4 was given over a period of 6 weeks by these workers and it is conceivable that such treatment damaged lymphocytes. There was no assessment of lymphocyte function in these reports other than by trypan blue exclusion. If the lymphocytes were damaged these results may merely reflect the accumulation of damaged lymphocytes in the liver (Zatz & Lance, 1970).

The preparation of T cell-depleted cell suspensions in this study was accomplished by the use of anti-θ serum and complement. The efficacy of this serum in removing the T cells was assessed by the PHA response. There was a marked reduction in the response to this mitogen following treatment of the cells with anti-θ serum and complement. As PHA is predominately a T cell mitogen (Callard, personal communication) this suggests that the treatment had been effective in removal of T cells. Moreover, in previous unrelated experiments this same batch of serum had been shown to abrogate other T cell responses (A. Basten, personal communication).

The production of T cell-deficient mice was shown also to be effective by the similar reduction in the PHA response observed in the thymectomized mice.

The ability to produce a similar degree of liver damage in both those animals who could mount a normal cell-mediated immune response and in those unable to do so suggests that the hepatic response to CCl_4 is not related causally to the lymphocyte response to liver antigens. These results are also consistent with the demonstrated inability to transfer the hepatic lesion to recipients of lymphocytes sensitized to liver antigens.

This data demonstrated that the observed lymphocyte response was mediated by T cells since the response was observed only in T cell-containing suspensions and could only be seen in T cell replete animals. In experimental hepatitis produced by the injection of allogeneic liver tissue to mice it has been suggested also that T cell sensitization to liver antigen is present (Warnatz *et al.*, 1975). Unlike the present study, the sensitized cells in the report by Warnatz *et al.* (1975) were felt to play a role in the pathogenesis of the hepatic lesion.

In many of the studies of human liver disease an antibody-dependent cell-mediated cytotoxicity (K cell) against rabbit hepatocytes has been observed (Cochrane *et al.*, 1976; Cochrane *et al.*, 1977). To demonstrate T cell cytotoxicity adequately requires HLA-compatible lymphocyte and target cells (Dickmeiss, Søeberg & Svejgaard, 1977) and thus in the studies in which K cell reactivity was demonstrated T cell cytotoxicity would not be observed even if cytotoxic T cells were present. In this study sensitized T cells were observed in the mice but it is not known whether such sensitized T cells are present in human liver disease patients. It is noteworthy that immune responses to LSP were not observed after paracetamol-induced toxic liver injury in man (Jensen *et al.*, 1978).

Although these studies are not able to be extrapolated directly to humans the conclusion can still be drawn that liver cell necrosis may result secondarily in T lymphocyte sensitization to liver antigen and the mere presence of such sensitized cells does not necessarily imply that the liver damage is caused by immune mechanisms.

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