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The effect of experimental iron-overload on splenic T cell function: analysis using cloning techniques

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SUMMARY

The effect of iron-overload on cell-mediated immunity was examined in C57 mice. Two methods of iron-loading were used: (i) dietary carbonyl iron which produced iron-loading primarily of parenchymal cells or (ii) intraperitoneal administration of iron-dextran which produced iron-loading predominantly of Kupffer cells. Both methods of iron-loading resulted in a diminished capacity of spleen cells to generate an allo-specific cytotoxic response in the absence of exogenous interleukin 2 (IL-2). Exogenous IL-2, however, restored the ability of spleen cells from iron-loaded mice to generate allo-specific cytotoxicity in bulk culture. Clonal assays for the precursor cells of cytotoxic T lymphocytes (CTL-P), performed in the presence of added IL-2, demonstrated that iron-loaded mice contained normal numbers of CTL-P. However, cultures of spleen cells from carbonyl iron-loaded mice generated less IL-2 following Concanavalin A stimulation, apparently as a result of a reduction in the number of IL-2-secreting cells amongst the spleen cell population. This work presents further evidence that iron-overload is associated with defective immunoregulatory control.

Keywords clonal analysis iron-loading techniques cellular immunity

INTRODUCTION

There is considerable evidence that both local (Richmond, 1959; Weinbren, Salm & Greenberg, 1978) and generalized (Berman, 1958; Powell, Mortimer & Harris, 1971; Weinberg, 1978; 1986; Anthoine *et al.*, 1979) iron-overload is associated with an increased incidence of infection and neoplasia. Both experimental and clinical iron-overload have been shown to be associated with a marked increase in neoplasia (Richmond, 1959; Nettesheim, Creasia & Mitchell, 1975; Bergeron, Streiff & Elliott, 1985); in genetic haemochromatosis (GH), the relative risk for hepatic neoplasia has recently been shown to exceed 200 (Bradbear *et al.*, 1985; Niederau *et al.*, 1985). Recurrent infections have been reported in patients with a variety of iron-overload disorders (haemochromatosis, sideroblastic anaemia, congenital dyserythropoietic anaemia and thalassaemia) (Van Asbeck *et al.*, 1982). In addition, *Yersinia enterocolitica* septicaemia and peritonitis have been reported in both adults and children with iron-overload (Rabson & Koornhoof, 1972; Rabson, Hallett & Koornhoof, 1975; Bouza *et al.*, 1980; Capron *et al.*, 1984; Boemi *et al.*, 1985).

The interaction between iron and the cellular immune system is complex and poorly understood.

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Iron and iron-binding proteins have been shown to impair *in vitro* a variety of immunological functions (De Sousa & Nishiya, 1978; Nishiya *et al.*, 1980; Matzner *et al.*, 1979; 1985) and also to interfere with non-specific defence mechanisms (Van Asbeck *et al.*, 1982). Bryan *et al.* (1981) noted that concentrations of ferric citrate as low as 0·1 mM resulted in a significant reduction of a one-way mixed lymphocyte reaction (MLR). Keown & Descamps-Latscha (1983) showed that both the iron-binding protein ferritin and Fe^{3+} could inhibit antigen-induced lymphocyte-proliferation and CTL sensitization while CTL effector function and mitogen presentation by monocytes were unaffected. Interleukin 2 (IL-2) partially abrogated the inhibitory effect of iron, suggesting that iron inhibited the production and release of IL-2 by helper cells.

In this study, we have analysed cytotoxic and helper T cell functions from normal and iron-loaded mice using both functional clonal assays for precursor cells, as well as analyses based on the phenotypic markers Ly-2 (Cantor & Boyse, 1975) and L3T4 (Dialynas *et al.*, 1983). Irrespective of the method of iron-loading, excess iron resulted in a significant alteration in the major functional subsets of splenic T lymphocytes.

MATERIALS AND METHODS

Animals

Sprague Dawley rats and BALB/c (H-2^d), C57BL6J (H-2^b) and CBA (H-2^k) male mice (7–12 weeks old) were obtained from the University of Queensland and The Animal Resources Centre (Willetton, Australia).

Experimental iron-loading techniques

Mice were iron-loaded either by administering three intraperitoneal injections of iron-dextran (5–15 mg) (Fisons Pty. Ltd, Sydney) over 2 weeks or by the addition of 0·5% carbonyl iron (GAF Corporation, New York, USA) to their diet for a period of 3–6 weeks (Bacon *et al.*, 1983). All animals were age-paired. Iron-dextran-loaded animals did not differ in weight from control animals, and carbonyl iron-loaded animals were, on average, 90% of the weight of control animals of similar age.

Estimation of hepatic iron

Hepatic iron concentration was measured by atomic absorption spectrophotometry (Varian Techtron Model AA6). A core of needle biopsy specimen of approximately 1 cm (4–8 mg wet weight) was assayed. After drying to constant weight, the specimens were washed with a mixture of concentrated sulphuric (0·5 ml), nitric (0·5 ml) and perchloric acids (0·2 ml). Samples were quantitatively transferred to acid-washed volumetric flasks and made up to volume with iron-free distilled water. The detection limit for iron was 0·005 μg/ml.

Culture medium

Liquid medium (H-MEM) contained 10 mM HEPES, sodium bicarbonate (3·6 g/l), penicillin (60 mg/l) and streptomycin (100 mg/l) in Eagle's minimal essential medium (Cat. 10–121., Flow Laboratories, Australia).

Preparation of murine lymphoid cell suspensions

Spleens and thymuses were removed under sterile conditions and gently teased through fine wire mesh into medium. Cell suspensions were washed three times in fetal calf serum (FCS). Cells were counted in a haemocytometer and Eosin dye exclusion was used as a marker of cell viability.

Target cells for cytotoxicity assays

Mouse peritoneal exudate cells (PEC) were used as targets. These were induced 5 to 8 days before use by an intraperitoneal injection of 2·5 ml of sterile Brewer's thioglycollate (40·5 g/l).

Preparation of concanavalin A-stimulated spleen cell conditioned medium (CAS)

Rat CAS was used as a source of IL-2 (Good & Nossal, 1983). Spleen cells (10^7 per ml) were

incubated (37°C, 2 h) with serum-free medium containing Concanavalin A (Con A) at 5 μg/ml and allowed to adhere. The supernatant was decanted, the adherent cells were washed twice and then incubated (37°C, 18 h) in the same volume of Con A-free medium. The supernatant medium (CAS) was centrifuged and stored at −20°C. CAS was filter-sterilized before use.

The ability of mouse spleen cells to secrete IL-2 following Con A stimulation was also tested using a similar protocol.

Cytotoxic lymphocyte cultures (MLC)

Bulk culture. Cytotoxic effector cells were generated as follows. Responder mouse spleen cells at $1{\cdot}5 \times 10^6$ per ml were co-cultured for 5 to 6 days with X-irradiated (2000 rads) allogeneic spleen cells at 2×10^6 per ml in H-MEM supplemented with 10% FCS and 5×10^{-5} M 2-ME in flat-bottomed Linbro trays in a volume of 1·5 ml per well. Some cultures also contained 10% CAS.

Micro MLC. Limiting numbers of responder mouse spleen cells were cultured for 7 days with 3×10^5 X-irradiated (2000 rads) allogeneic spleen cells in 0·2 ml H-MEM supplemented with 10% FCS, 10% CAS and 5×10^{-5}M 2-ME in 96-well V-bottomed plates. Three columns (24 wells per plate) were control wells lacking responder cells.

Analysis of lytic potential

Cytotoxic T lymphocytes (CTL). These were generated in bulk culture, as described. These were harvested and viable cell counts were determined. Effector cells were assayed by a 4 h incubation at different effector to target ratios in Linbro plates (Cat. 76-013-05., Flow Laboratories, Sydney, Australia). Quadruplicate assays were performed with 5×10^4 PEC targets per well in a volume of 0·2 ml. Lysis was determined by the method of Parish & Mullbacher (1983) with minor modifications, as follows. After incubation, non-adherent cells and culture medium were flicked off the plates and 10 μl of Neutral Red (0·036%) in phosphate-buffered saline (PBS) was added to each well. After incubation for 15 min at 37°C, the plates were washed three times in 0·15 M sodium chloride and viable adherent PEC (containing Neutral Red) were determined by adding 100 μl of a 50/50 mixture of ethanol and 100 mM acetic acid. Wells that did not contain cytotoxic activity were bright red. The absorbance of each well was determined using a wavelength of 540 nm and a reference wavelength of 520 nm. Control wells contained PEC to which cultured irradiated allogeneic cells from culture were added for the 4 h incubation. Background wells contained only irradiated allogeneic cells from culture. These wells were used to obtain control and background absorbance values. Specific lysis was calculated from the equation:

$$\text{Specific lysis} = \frac{\text{control absorbance} - \text{experimental absorbance}}{\text{control absorbance} - \text{background absorbance}}$$

From specific lysis values, lytic units were determined. One lytic unit was defined as the number of effector cells required to lyse 50% of targets.

Micro MLC. Each microculture was assayed for its capacity to lyse 5×10^4 PEC targets over a 16 h incubation. After Neutral Red staining, the absorbance of each of the control and experimental wells was determined. Positive wells were defined as having an absorbance less than 3 s.d. below the mean absorbance of the control wells.

Culture of IL-2-producing precursor cells

A clonal assay for helper T lymphocyte-precursors was performed using the method of Hefeneider *et al.* (1984). Limiting numbers of spleen cells were co-cultured with 3×10^3 syngeneic PEC (non-induced) as a source of interleukin 1 in 0·2 ml H-MEM with 10% FCS, 5×10^{-5}M 2-ME and concanavalin A (Con A) at 5 μg/ml. Control wells contained no spleen cells.

Assessment of IL-2-producing clones

After five days culture, 4000 cells of an IL-2 dependent cell line, CTLL (Gillis *et al.*, 1978), were added to each well. Eighteen hours later, the wells were pulsed with 0·5–1 μCi of ^{3}H-thymidine. Wells in which CTLL proliferation had occurred (positive wells) were defined as those in which incorporation of ^{3}H-thymidine exceeded the mean of control wells by more than 3 s.d.

Assessment of IL-2 concentration

IL-2 concentration was measured using a biological assay (Gillis *et al.*, 1978). One unit per ml was defined as the concentration of IL-2 that supported to 50% of maximum, the proliferation of CTLL during an overnight incubation when CTLL were present at 4000 cells per well in a volume of 0·2 ml. Quadruplicate assays were performed. The dilution factor of the sample required to give 1 unit per ml was defined as the number of units per ml of IL-2 in the sample.

Precursor frequency estimation

Cytotoxic T lymphocyte-precursor (CTL-P) frequencies were determined using a least squares formula to plot cells per well against the logarithm of the fraction of non-cytotoxic microcultures (line-plot). Precursor frequencies for IL-2-secreting cells were determined by plotting cells per well against the logarithm of the fraction of non-IL-2-secreting cultures (line-plot) (Lefkovits & Waldman, 1979).

Phenotypic analysis

Phenotypic analyses were performed using monoclonal antibodies to surface markers that identify murine cytotoxic and helper cells (Ly-2 and L3T4) respectively (Cantor & Boyse, 1975; Dialynas *et al.*, 1983). The percentage cell killing following treatment with antibody and complement was compared with that following treatment with complement alone.

Statistical tests

A paired *t*-test (Winer, 1971) was used to calculate the statistical significance between results from correlated groups.

RESULTS

Carbonyl-iron-loading reduces the ability of spleen cells to generate CTL. Adult C57 mice, iron-loaded by dietary carbonyl iron (0·5%), showed storage of iron predominantly in hepatocytes. After 3–6 weeks, spleen cells from these mice and from control mice were stimulated in a bulk mixed lymphocyte culture with irradiated allogeneic cells (BALB/c) in the absence of exogenous IL-2. Figure 1 illustrates the results from one such experiment and the method of determination of lytic units. Cytotoxicity and lytic units were determined after 5 days.

Table 1 gives results for four consecutive experiments. As shown for all experiments, there was a significant reduction in the number of lytic units generated from spleen cells from iron-loaded mice.

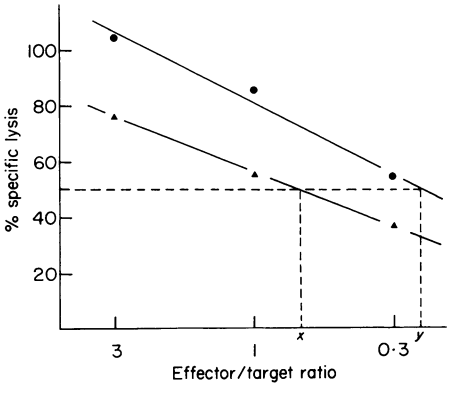

Fig. 1. Derivation of lytic units. CTL were generated using spleen cells from either iron-loaded (▲) or control mice (●), and tested for specific cytotoxicity at different effector to target ratios. A lytic unit is defined as the number of effector cells required to generate 50% specific lysis.

Table 1. Immunoregulatory effects of iron-overload (carbonyl iron)

Expt. number	C57 Mice	Lytic units/ culture*	Lytic units/ spleen†	Splenic CTL-P frequency ($\times 10^6$)	IL-2 Production by spleen cells‡	Lytic Units/ IL-2+Con A supernatants§
1	Fe-Loaded	110	779	638	29·4	131
	Control	177	1358	553	37·0	143
2	Fe-Loaded	37	220	583	6·5	756
	Control	125	1057	516	13·7	282
3	Fe-Loaded	54	486	541	3·9	1377
	Control	400	1911	563	5·3	528
4	Fe-Loaded	277	1571	1534	8·2	319
	Control	459	2700	1421	9·3	267
	P value	<0·05	<0·01	NS	<0·05	NS

The mean hepatic iron concentration for the iron-loaded mice was 35 μmol/g dry weight with s.e.m. = 4·9 μmol/g, and 3·83 μmol/g for the control animals with s.e.m. = 0·43 μmol/g.

* CTL were generated *in vitro* from C57 (H-2^b) responder spleen cells reacting to BALB/c (H-2^d) stimulators.

† C57 anti-BALB/c CTL-P frequencies were calculated using limit-dilution analysis (for example, see Fig. 2).

‡ IL-2 was produced *in vitro* following Con A stimulation of spleen cells. Units given on a per spleen basis.

§ Supernatant from Con A-stimulated spleen cells, containing 10 units/ml of IL-2, was present during generation of CTL *in vitro*.

Clonal analysis. The reduced cytotoxic potential of spleen cells from iron-loaded mice could not be accounted for by the presence of fewer allo-specific cytotoxic T lymphocyte precursors (CTL-P) present in the spleen as determined by limit-dilution (clonal) experiments, as the frequency of CTL-P did not differ between Fe-loaded and control animals (Fig. 2 and Table 1). Since iron-loading did not affect splenic size in these experiments, it was concluded that the spleens of iron-loaded mice contained normal numbers of CTL-P.

Spleen cells from iron-loaded and control mice were also stimulated with Con A so that IL-2 production could be assessed (Table 1). There was a significant reduction ($P < 0·05$) in the ability of spleen cells from iron-loaded mice to generate IL-2. In one experiment (No. 2), a limit-dilution analysis of the precursors of IL-2-secreting cells was performed, as described in Materials and Methods (Fig. 3). There were $44·3 \times 10^3$ precursors per spleen in the control mice and $20·9 \times 10^3$ in the iron-loaded group. This ratio was similar to that derived from the measurement of units of IL-2 secreted in bulk culture, indicating that the decreased T-help in iron-loaded mice could be accounted for by a reduction (functional or actual) of precursor cells in the spleen.

Helper T-cell factors restore the lytic capacity of spleen cells from iron-loaded mice. That the reduction in lytic capacity was due to a reduction in T cell help was determined by restoring with helper T cell factors (supernatant from Con A-stimulated spleen cells) the capacity of spleen cells from iron-loaded mice to generate cytotoxicity in bulk culture (Table 1). This result was supported by the limit-dilution analysis for CTL-P, where exogenous IL-2 was present in the microcultures (Table 1).

Phenotypic analysis demonstrated that spleen cells from iron-loaded mice contained significantly fewer cells that were lysed by anti-L3T4 and complement ($P < 0·005$), indicating that iron-loading caused an actual reduction of HTL-P in the spleen. No significant difference was observed in the presence of anti-Ly2.

Iron-dextran loading reduces the ability of spleen cells to generate cytotoxicity. Further studies

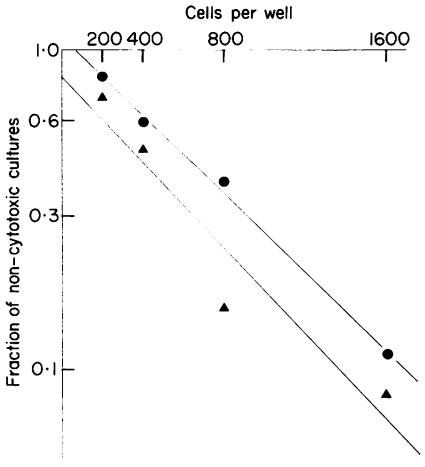

Fig. 2. CTL-P limit-dilution analysis of spleen cells from either iron-loaded (▲) or control (●) mice. Limiting numbers of spleen cells were co-cultured with irradiated allogeneic spleen cells for 7 days. Individual cells were then tested for cytotoxicity.

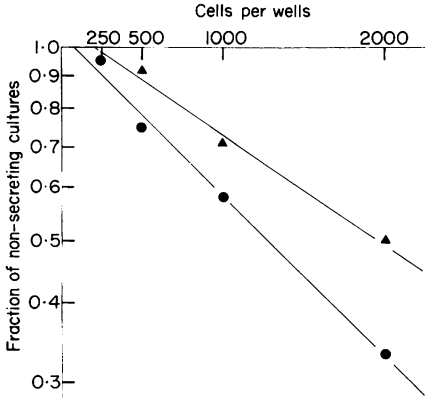

Fig. 3. Limit-dilution analysis of spleen cell precursors for IL-2 secretion from either iron-loaded (▲) or control (●) mice. Limiting numbers of spleen cells were co-cultured with 3,000 PEC per cell in the presence of Con A for 5 days. Supernatants were then screened for the presence of IL-2.

were carried out with BALB/c, C57 and CBA mice which were iron-loaded by the intra-peritoneal administration of iron-dextran, resulting in iron-overload predominantly in Kupffer cells. Mice used as controls received injections of saline. Spleen cells from iron-loaded animals again generated less specific cytotoxicity than spleen cells from control animals (Table 2). In two of the experiments (Numbers 6 and 7), cell mixing studies were performed. Cells from iron-loaded and control animals were mixed 50/50 before culture and the results demonstrated that cellular suppression was not contributing to the reduced cytotoxicity.

Limit-dilution experiments revealed that the spleens of iron-dextran-loaded mice, like those of carbonyl iron-loaded mice, contained normal numbers of CTL-P.

DISCUSSION

This study has shown that iron overload produced by either dietary or parenteral means was

Table 2. Spleen cells from iron-loaded mice (iron-dextran) generate less cytotoxicity in bulk culture

Expt. number	Mice	Lytic units/culture		
		Fe-loaded	Control*	Fe/C (50/50)
5	BALB/c anti-CBA	14·3	147·5	—
6	BALB/c anti-CBA†	14·5	126·2	168·8
7	BALB/c anti-C57†	0·5	1·9	1·7
8	C57 anti-BALB/c	5·5	19·9	—
9	C57 anti-CBA	13·3	18·1	—
10	CBA anti-BALB/c	16·4	184·0	—
11	CBA anti-C57	37·1	261·8	—
		$P < 0{\cdot}025$		

Mice were iron-loaded by intra-peritoneal administration of iron-dextran. Hepatic iron concentrations of all iron-loaded mice (Fe) were similar (mean = 119 μM/g dry weight, s.e.m. = 5·5)

* Control mice (C): mean = 3·7 μM/g, s.e.m. = 0·63.

† In mixing experiments, cells from iron-loaded (Fe) and control (C) mice were mixed 50/50 before culture.

associated with a reduction in the ability of spleen cells to mount an allogeneic cytotoxic response. Injections of iron-dextran resulted in iron present chiefly in the Kupffer cells, whereas in mice fed dietary carbonyl iron, iron was deposited mainly in the hepatocytes in a periportal distribution, which closely resembled that seen in idiopathic haemochromatosis (Bacon *et al.*, 1983).

The reduced cytotoxicity could result theoretically from a reduction in CTL-P, a reduction in T-cell help or from the effect of suppressor cells. Clonal experiments permitted the differentiation between these possibilities. In four separate experiments, frequencies of allo-specific CTL-P in the spleens of carbonyl iron-loaded animals were not different from the controls. To estimate T-cell help, IL-2 production was measured following stimulation with Con A. IL-2 production by spleen cells of iron-loaded animals was moderately reduced. This reduction correlated closely with the reduction in the number of spleen cells capable of secreting IL-2 (Fig. 3). Exogenous helper cell factors when added to bulk cultures of spleen cells from iron-loaded animals restored the level of cytotoxicity. This finding was also demonstrated by CTL-P limit-dilution cultures which contained exogenous IL-2. To exclude the possibility that iron-loading affected the production of IL-1 by monocytes, normal peritoneal exudate cells were added to provide a source of IL-1 in the precursor assay for IL-2 secreting spleen cells (Fig. 3).

The reduced numbers of functional precursors of IL-2 secreting cells in iron-loaded animals could result from: (1) a functional (anergic) or actual deletion of helper T cell precursors; (2) a redistribution of these precursors to other sites; or (3) their delayed ontogeny. Phenotypic studies using the specific T-helper cell marker L3T4 (Dialynas *et al.*, 1983) suggested that fewer helper precursors were present in the spleens of iron-loaded mice. This would indicate that there was not merely a functional deletion of helper precursors. The present experiments do not permit a distinction between the other possibilities mentioned above. However, mice were studied within a few weeks following iron-loading. It would thus be unlikely that an arrest in the ontogeny of helper precursors could be responsible for the findings.

It is noteworthy that iron-loading by either dietary carbonyl iron or parenteral administration of iron-dextran affected splenic lymphocyte distribution. In such mice histological examination revealed grade 3/4 iron-loading in the liver without evidence of hepatitis, cirrhosis or splenomegaly. A possible explanation for the observed immunological derangement in both groups of iron-loaded mice is that even moderate iron-loading has been shown to be associated with an increase in serum non-transferrin-bound iron (Hershko *et al.*, 1978; Batey *et al.*, 1980; Gutteridge *et al.*, 1985), which

has been shown to modulate lymphocyte function (Van Asbeck *et al.*, 1982; Matzner *et al.*, 1979; 1985). In the study by Van Asbeck *et al.* (1982), the occurrence of *Listeria monocytogenes* meningitis in an iron-overloaded patient with haemochromatosis was reported. It was observed that serum from this patient, as well as ferric citrate, but not sodium citrate or ferritin, inhibited phagocytic cell function and that therapeutic phlebotomy was able to reverse completely this effect of the serum.

The present study suggests that in iron-overload, T cell help is reduced in the spleen. Both experimental models used in this study produced increased iron concentration in the spleen, as seen in secondary iron-overload in humans. It is noteworthy that our experiments *in vitro* have been performed in the absence of added iron. Iron-overload may inhibit the generation of an immune response by: (1) reducing available T cell help in the spleen; and (2) by directly inhibiting the generation of effector cells. These complementary effects would severely limit a T cell response, and this, in conjunction with the high degree of saturation of serum transferrin which can be associated with the detection of non-transferrin-bound iron in the serum (Gutteridge *et al.*, 1985), may offer a possible explanation for the predisposition of iron-loaded subjects to infection and neoplasia.

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