Immunology 1981 43 387

The effect of iron and transferrin on the response of serum-free cultures of mouse lymphocytes to Concanavalin A and lipopolysaccharide

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Accepted for publication 12 January 1981

Summary. Incorporation of [^{14}C]-thymidine into mouse lymph node cells stimulated with either Concanavalin A or lipopolysaccharide in serum-free medium was markedly enhanced by the addition of transferrin. Thymidine incorporation was similar in transferrin-containing serum-free medium and in medium containing 5% foetal calf serum. Transferrins from both homologous and heterologous species were equally effective, but iron-binding half-molecules of transferrin, and low molecular weight iron chelates produced no enhancement. The optimal response was obtained with 10–50 μg/ml of transferrin, and with 30%–70% iron saturation. Although the major function of transferrin in lymphocyte cultures is probably to supply iron, it may also fulfil other functions.

INTRODUCTION

There is a considerable amount of clinical evidence indicating that iron deficiency may be associated with depression of immune responsiveness (see review of Jacobs, 1977). A possible explanation of these observations may lie in an inability of lymphocytes of patients with iron-deficiency to obtain sufficient iron for the burst of metabolic activity associated with transformation. Not a great deal is known about the iron requirements of lymphocytes, or of other non-erythroid cells, although a growing number of recent reports indicate that the iron-transport protein transferrin is an essential requirement for the *in vitro* proliferation of a variety of non-erythroid cells in serum-free medium (Rudland, Durbin, Clingan & Jiménez de Asua, 1977; Iscove & Melchers 1978; Barnes & Sato 1979; Breitman, Collins & Keene, 1980). In particular, Phillips & Azari (1975) have shown that thymidine incorporation into phytohaemagglutinin (PHA)-stimulated human lymphocytes cultured in serum-free medium is enhanced by transferrin.

In this paper the role of transferrin and iron in the response of mouse lymph node cells to Concanavalin A (Con A) and lipophysaccharide (LPS) has been investigated. A serum-free medium has been employed to permit control of iron and transferrin levels. In the following paper (Brock & Rankin 1981) the ability of the proliferating cells to bind transferrin and take up iron has been examined.

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0019-2805/81/0600-0387$02.00
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MATERIALS AND METHODS

Transferrins and transferrin fragments

Human transferrin was purchased from Sigma (Poole, Dorset) or from Behringwerke Hoechst, Hounslow,

Middx. Both preparations were iron-free, and gave similar results. Bovine transferrin was prepared as described previously (Brock, Arzabe, Lampreave & Piñeiro 1976), and a similar procedure was used to obtain mouse transferrin from pooled mouse serum, except that the initial rivanol-precipitation step was omitted. Monoferric fragments of bovine transferrin were obtained by tryptic cleavage of bovine Fe_2-transferrin (Brock & Arzabe, 1976). Each fragment corresponds to approximately half the transferrin molecule, and conains either the N- or C-terminal iron binding site (Brock & Arzabe, 1976; Brock, Arzabe, Richardson & Deverson, 1978).

Lymphocyte cultures

Cell suspensions were prepared from the peripheral and mesenteric nodes of adult C3H mice. The fact that mesenteric nodes contain a small percentage of blast cells was shown in preliminary experiments not to influence results, which did not vary significantly when cells from only one or other type of node were used. The suspensions contained approximately 95% lymphocytes and were 70%–80% viable as measured by eosin exclusion. The cells were washed once in RPMI 1640 medium containing 25 mM HEPES (Flow Laboratories, Irvine, Ayrshire) and 1 ml cultures containing 2×10^6 viable cells were set up in acid-washed 10 cm × 1 cm glass test tubes in RPMI 1640 medium (without HEPES), supplemented with 50 μM 2-mercaptoethanol and either 1 mg/ml human serum albumin (Behringwerke) for serum-free medium, or with 5% foetal calf serum (Flow Laboratories). The albumin contained no detectable transferrin. Preliminary experiments showed that omission of either of the supplements from the serum-free medium, resulted in about 50% reduction in thymidine incoporation. Con A (Miles-Yeda, Israel; 1 μg/ml in serum-free cultures or 8 μg/ml in serum-containing cultures) and lipopolysaccharide from *Salmonella typhimurium* (Difco; 5 μg/ml) were added as required. Human transferrin was normally added at 50 μg/ml and 30% iron saturation achieved by adding 15 ng/ml of iron as the ferric nitrilotriacetate complex. In some experiments 1 mM desferrioxamine (Desferal, Ciba-Geigy) was also added. The iron content of the culture medium, before adding exogenous iron, was estimated by carbon furnace atomic absorption spectroscopy and found to be 6 ng/ml. Cultures were incubated for up to 72 hr in 10% CO_2/90% air mixture at 37° in a gas box.

DNA synthesis. Cultures were pulsed for 4 hr with 0·05 μCi of [^{14}C]-thymidine (The Radiochemical Centre, Amersham) and the cells harvested on glass-fibre mats which were then washed with 10% (w/v) trichloroacetic acid and methanol. After drying the mats were counted in a Packard Model 3320 scintillation counter to assess DNA synthesis.

RESULTS

Effect of iron and transferrin on the response of mouse lymph node cells to mitogens

Addition of transferrin to the serum-free culture medium markedly enhanced the response to both Con A (Fig. 1) and LPS. Little thymidine incorporation occurred in the absence of mitogens, indicating that transferrin was not acting as a mitogen *per se*. The response in transferrin-containing serum-free medium was similar to that seen in media containing 5% foetal calf serum, though background DNA synthesis in the absence of mitogens was some 42% less with serum-free medium, presumably due to the elimination of mitogenic factors often present in foetal calf serum (Table 1). It should also be noted that optimal

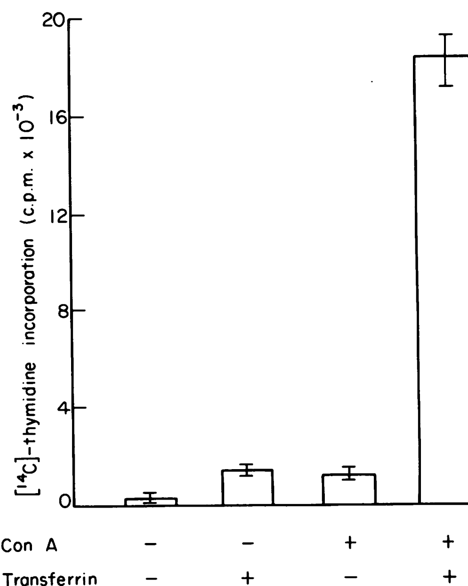

Figure 1. [^{14}C]-thymidine incorporation into mouse lymph node cells cultured for 72 hr in serum-free medium in the presence or absence of Concanavalin A (1 μg/ml) and human transferrin (50 μg/ml; 30% iron-saturated). Mean ± standard deviation, $n = 3$).

Table 1. Effect of serum and transferrin on [^{14}C]-thymidine incorporation into mouse lymph node cells stimulated with Con A or LPS

	[^{14}C]-thymidine incorporation (c.p.m.)		
RPMI 1640 medium supplemented with	Con A*	LPS (5 μg/ml)	No mitogen
Albumin (1 mg/ml)	1410	1252	373
Albumin (1 mg/ml) + transferrin (50 μg/ml, 30% Fe-saturated)	17,038	10,127	1410
5% foetal calf serum	17,075	11,081	2428

All media contained 50 μM 2-mercaptoethanol.
* 1 μg/ml in serum-free medium, 8 μg/ml in medium containing foetal calf serum.

responses to Con A were obtained with 8 μg/ml in serum-containing medium, but with only 1 μg/ml in serum-free medium. This difference is probably due to competition by serum glycoproteins for binding of Con A when foetal calf serum is present.

Effect of low molecular weight iron chelates

When nitrilotriacetate or citrate complexes of iron were substituted for iron transferrin the response to Con A was similar to that seen in controls containing neither iron nor transferrin (Fig. 2). When desferrioxamine was added to cultures containing iron and transferrin, thymidine incorporation was almost totally inhibited. Low molecular weight iron chelates therefore cannot substitute for iron-transferrin, and furthermore the high affinity chelator desferrioxamine interferes with the enhancing effect of iron-transferrin.

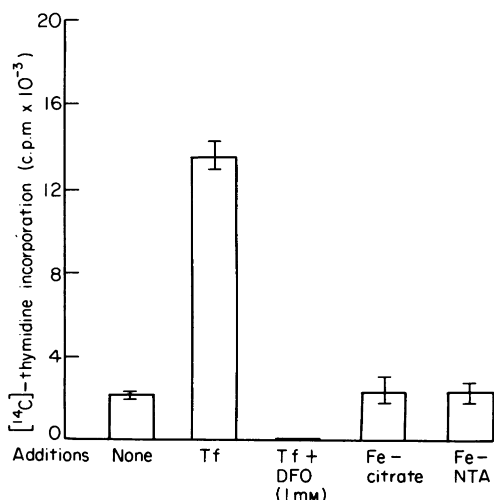

Figure 2. [^{14}C]-thymidine incorporation into mouse lymph node cells cultured for 72 hr in serum-free medium in the presence of Concanavalin A (1 μg/ml) and various iron compounds. The iron concentration was 15 ng/ml in all cases, giving approximately 30% saturation of transferrin (Tf). DFO = desferrioxamine (1 mM); NTA = nitrilotriacetate. Mean ± standard deviation (n = 3).

Specificity of transferrin requirement

Mouse, human and bovine transferrins were all effective in enhancing the response of mouse lymph node cells to Con A (Fig 3). Monoferric fragments of bovine

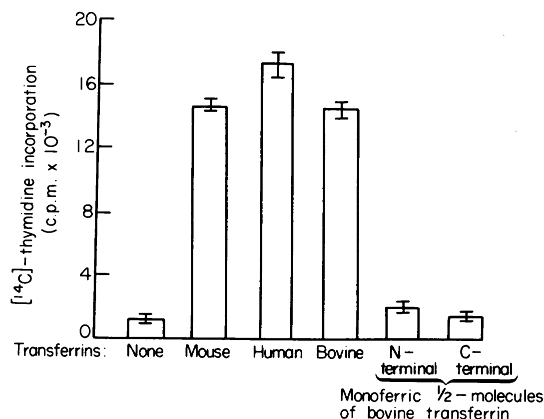

Figure 3. [^{14}C]-thymidine incorporation into mouse lymph node cells cultured for 72 hr in serum-free medium in the presence of Concanavalin A (1 μg/ml) and transferrins from various species, and monoferric fragments of bovine transferrin. Transferrins and fragments were added at 50 μm/ml and 30% iron saturation. Mean ± standard deviation (*n* = 3).

transferrin were, however, ineffective. Thus although the requirement for transferrin can be satisfied by proteins from both the homologous species (mouse) or heterologous species (human and bovine), cleavage of the molecule is not tolerated even if the iron-binding sites remain intact. The equal response to both mouse and human transferrins would seem to justify the use of the latter routinely, rather than mouse transferrin which is not readily available.

Effect of transferrin concentration

Optimal enhancement of [^{14}C]-thymidine incorporation into lymphocytes stimulated with Con A was achieved by adding 10-50 μg/ml of transferrin, but even 0·5 μg/ml produced considerable enhancement (Fig. 4). It should be noted that although the transferrin added was in each case approximately 20% saturated with iron, the iron content of the medium (6 ng/ml) becomes increasingly significant as the transferrin concentration decreases, and is sufficient to saturate the lowest amounts used. As shown below, this in itself may have affected the proliferative response.

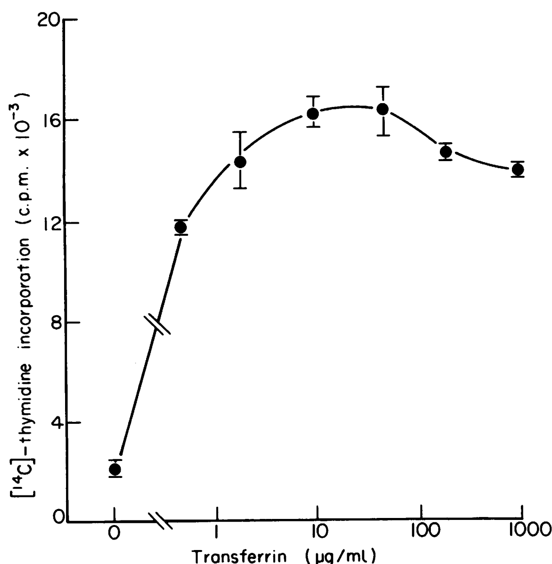

Figure 4. [^{14}C]-thymidine incorporation into mouse lymph node cells cultured for 72 hr in serum-free medium in the presence of Concanavalin A (1 μg/ml) and various concentrations of transferrin (30% iron-saturated). Mean ± standard deviation ($n=3$).

Effect of iron saturation

In experiments in which the transferrin concentration was maintained at 50 μg/ml but iron saturation was varied it was found that optimal enhancement of the response to Con A occurred at saturation between 30% and 70% (Fig.5). Fully saturated transferrin was markedly less effective, as also was 8% saturated transferrin. This last was achieved by adding apotransferrin directly to the medium, and the resulting 8% saturation was due to the iron content of the culture medium. The effect of totally iron-free transferrin was therefore tested indirectly by adding desferrioxamine to the medium to chelate endogenous iron before adding apotransferrin. Since the affinity of desferrioxamine for iron is greater than that of transferrin, iron bound by desferrioxamine cannot subsequently be bound by transferrin. It was found that the enhancing effect of transferrin on DNA synthesis was reduced when the amount of desferrioxamine added exceeded that necessary for chelation of the endogenous iron (Fig. 6). Thus iron-free transferrin is probably ineffective in enhancing the response of mouse lymphnode cells to Con A.

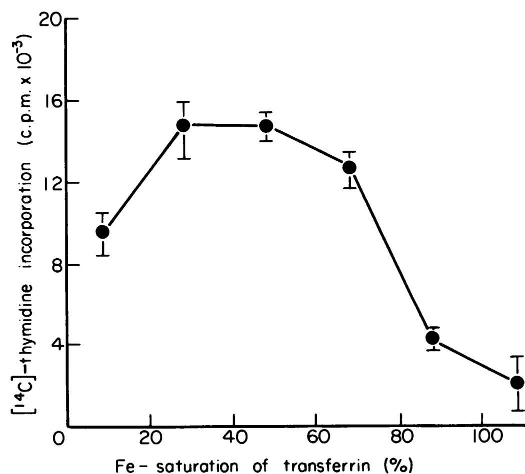

Figure 5. [^{14}C]-thymidine incorporation into mouse lymph node cells cultured for 72 hr in serum-free mdium in the presence of Concanavalin A (1 μg/ml) and 50 μg/ml transferrin, of varying iron saturation. Mean ± standard deviation ($n=3$).

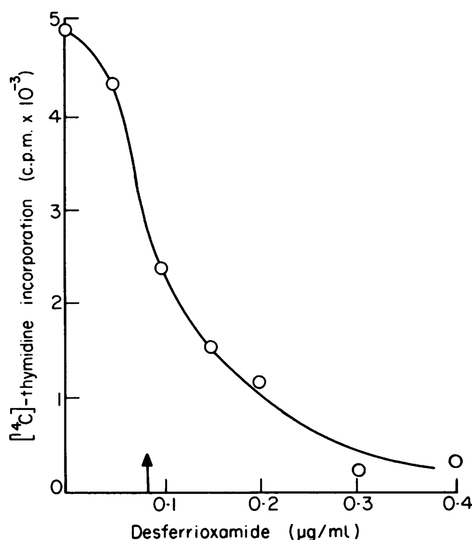

Figure 6. The effect of desferrioxamine (DFO) on [^{14}C]-thymidine incorporation into mouse lymph node cells cultured for 72 hr in serum-free medium in the presence of Concanavalin A (1 µg/ml) and apotransferrin (50 µg/ml). The arrow represents the minimum amount of DFO required to bind the endogenous iron present in the culture medium (8 ng/ml).

DISCUSSION

From the results presented in this paper it is clear that proliferation of mouse lymphocytes in response to mitogens requires both transferrin and iron, and that neither alone will suffice. These results confirm and extend the findings of Phillips & Azari (1975) who reported an enhanced response of human lymphocytes to PHA when iron-transferrin was added to a serum-free medium. Similar results have been reported by Dillner-Centerlind, Hammerström & Perlman, (1979) who found that the enhancing effect of some preparations of albumin was due to contamination with transferrin. In the present study highly purified transferrin-free albumin was routinely used to supplement the serum-free medium. The specificity of the requirement for transferrin was further demonstrated by the inability of monoferric half-molecules of bovine transferrin to enhance the proliferative response, although both homologous (mouse) and heterologous (human and bovine) transferrins were effective. These results parallel earlier studies on the uptake of transferrin-bound iron by reticulocytes in which it was found that heterologous transferrins could donate iron to rabbit reticulocytes, but monoferric fragments could donate iron only extremely slowly (Esparza & Brock, 1980a, b) and would support the concept that the major function of transferrin in lymphocyte proliferation is to supply iron. Chelation of toxic cations, suggested by Iscove & Melchers (1978) as an alternative function, appears to be at most a subsidiary mode of action, since monoferric fragments would also chelate such cations, yet were ineffective in enhancing proliferation.

It should, however, be noted that transferrin produced an optimal effect when the iron saturation was in the region of 30%–70%, rather than fully saturated. Whilst it is posible that these results might indicate that lymphocytes react preferentially with half-saturated rather than fully-saturated transferrin, it is perhaps more likely that transferrin performs aditional functions such as supplying zinc (Phillips, 1978) or, as mentioned above, binding toxic cations. Since Fe^{3+} is bound with greater affinity than any other cation, saturation of transferrin with iron would abrogate either of these other functions. Such an effect of iron saturation was not found by Phillips & Azari (1975) or by Dillner-Centerlind *et al.* (1979) who however, only examined the effects of either almost iron-free or iron-saturated transferrins, neither of which, in the present study, gave optimal enhancement of the response to Con A (Fig. 5).

The reduced ability of transferrin of $<10\%$ iron saturation to support lymphocyte stimulation suggests that in iron-deficiency where transferrin saturation is frequently below 10%, impairment of lymphocyte transformation may occur and contribute to the reported lowering of cell-mediated immune responses.

The ability of transferrin, together with albumin, to largely replace the requirement for serum in lymphocyte transformation and the requirement for transferrin in proliferation of a number of other non-erythroid cell types in serum-free media (Rudland *et al.*, 1977; Barnes & Sato, 1979; Breitman *et al.*, 1980) suggests a function for transferrin common to many cell types. In the following paper (Brock & Rankin 1981) we show that in the case of transforming mouse lymphocytes, transferrin acts as an iron donor to the proliferating cells.

ACKNOWLEDGMENTS

I thank Dr D.I. Stott for providing lipopolysaccharide and Dr G. Fell, Glasgow Royal Infirmary, for estimating the iron content of the serum-free medium. This paper is Department Publication No. 8015.

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