

# Inhibition of Human Lymphocyte Proliferation by Monoclonal Antibody to Transferrin Receptor

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**A monoclonal antibody, 42/6, which blocks the binding of transferrin to its receptor on the cell membrane, inhibits proliferation of human lymphocytes stimulated by phytohemagglutinin. Anti-receptor antibody B3/25, which does not block transferrin binding, does not alter the mitogenic response. Addition of soluble iron, in the form of ferric nitrilotriacetic acid, results in partial reversal of inhibition.**

**Lymphocytes in the quiescent phase of the cell cycle at the time of 42/6 antibody addition are unable to traverse S phase, whereas cells actively proliferating when antibody is added are sensitive to its inhibitory effects throughout all phases of the cell cycle. Inhibition is static rather than cidal, since it can be reversed by removal of antibody after up to 48 hr of exposure.**

**T**HE USE OF monoclonal antibodies (MoAbs) against cell surface membrane antigens to regulate cell proliferation and function is under active investigation in many laboratories. Antibodies against receptors for hormones and transmitter molecules exist in nature and have been shown to be involved in the pathogenesis of a number of human diseases.<sup>1</sup> The best known examples are myasthenia gravis, certain forms of insulin resistance, and thyroid disease associated with long-acting thyroid stimulator (LATS). With this background in mind, we began a series of studies designed to test applicability of monoclonal antibodies against hormone and growth factor receptors as useful modulators of cell proliferation and function. Initial studies by one of us (I.T.) examined the effects of MoAb against the transferrin receptor (TfR) on permanent and malignant cell lines.<sup>2-4</sup> The present study explores effects on the proliferation of normal human lymphocytes stimulated by phytohemagglutinin (PHA) in primary culture.

The requirement for transferrin in serum-free cultures of lymphocytes was established first in studies with murine B cells.<sup>5</sup> Subsequent investigations in a number of laboratories, including ours,<sup>6</sup> have established that human T lymphocytes cultured under serum-free conditions require supplementation with transferrin, insulin, and a source of fatty acids. Albumin is needed for optimal growth, and other molecules, such as selenium and ethanolamine, have the capacity to promote proliferation in serum-free cultures.<sup>6</sup>

The availability of MoAb that blocks binding of transferrin to its receptor has allowed us to examine the requirement of human T cells for transferrin in standard serum-supplemented culture conditions. By adding MoAb for varying intervals and by examining cell cycle distribution patterns and population growth, we have gained insight into the effects of transferrin on proliferation kinetics.

## MATERIALS AND METHODS

### Preparation of Lymphocytes

Lymphocytes were obtained from the peripheral blood of healthy normal donors, who gave informed consent. The method for isolating

a mononuclear cell population containing approximately 70% T lymphocytes, 15% monocytes, 10% B lymphocytes, and 5% null cells has been described previously.<sup>7</sup> Peripheral blood was defibrinated by agitation with glass beads and allowed to sediment in 1% dextran for 1 hr. Further purification was obtained by isopycnic centrifugation over Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ), followed by three washes in RPMI medium (Irvine Scientific, Santa Ana, CA).

### Culture Conditions

Cells were grown in 1-ml cultures at an initial concentration of  $4-6 \times 10^5$ /ml in RPMI medium supplemented with 10% autologous serum, 2 mM glutamine, and 1% Fungi-Bacti antibiotic mixture (Irvine Scientific). PHA-P (Burroughs-Wellcome, Research Triangle Park, NC) was added at a final concentration of 1  $\mu$ g/ml, which promotes optimal proliferation. Ferric nitrilotriacetic acid was prepared as described<sup>8</sup> and was added to the medium at the initiation of some experiments.

### Proliferation Assays

Cell proliferation was measured by cell counts using a hemacytometer, and viability was determined by the trypan blue exclusion method.<sup>9</sup> DNA synthesis was assayed indirectly by measuring <sup>3</sup>H-thymidine incorporation (2  $\mu$ Ci/ml, 6 Ci/mM) (Swartz Mann, Orangeburg, NY) during a 2-hr incubation. To measure cell cycle kinetics, cells grown in the presence or absence of antibodies were fixed at various time points in 70% ethanol, washed in 1.1% sodium citrate, treated with RNase for 30 min, and incubated with propidium iodide (0.025  $\mu$ g/ml).<sup>10</sup> Fluorescence was determined on an Ortho Cytofluorograph, 50H (Ortho Diagnostic Systems, Westwood, MA).

### Binding Studies

Binding of MoAb to lymphocyte surface membrane was assessed by cytofluorometry, as described previously.<sup>11,12</sup> Nonstimulated con-

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control cultures and 24-hr and 96-hr cultures of PHA-stimulated lymphocytes were washed 3 times in phosphate-buffered saline (PBS), pH 7.4, containing 0.1% azide, then incubated with MoAb for 30 min on ice, again washed 3 times with PBS-azide, and subsequently incubated 30 min with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Antibodies Incorporated, Davis, CA). After 3 more washes, cells were fixed in 1% formalin and analyzed on the Ortho cytofluorograph. A 5-W argon ion laser source (Lexel Corp., Palo Alto, CA) was used at 488 nm for fluorescence activation and for generation of light scatter data. Simultaneous measurements of forward angle light scatter and right angle light scatter were used to electronically gate out nonviable lymphocytes and cell clumps.<sup>13</sup> A fluorescence histogram for single viable lymphocytes was generated over an intensity range of 1,000 channels. Control data for nonspecific fluorescence were generated by reacting lymphocytes with an irrelevant mouse myeloma protein, RPC5 (Litton Bionetics Laboratories, Kensington, MD) and assessing binding of FITC-labeled goat anti-mouse IgG in an identical manner.

### MoAb

The MoAbs used in these experiments have been characterized previously. B3/25 anti-TfR MoAb is an IgG, which binds to the TfR molecule but does not block the binding site for transferrin.<sup>3</sup> 42/6 MoAb is an IgA, which binds to the TfR with higher affinity than transferrin itself and inhibits binding of transferrin.<sup>4</sup> T29/33 MoAb is an IgG, which binds to T200 glycoprotein, a major leukocyte cell surface glycoprotein.<sup>14</sup> T101 MoAb (gift from Dr. Ivor Royston) is an IgG specific for a T-lymphocyte cell surface antigen.<sup>11</sup>

## RESULTS

### Binding of MoAb to Human Peripheral Blood Lymphocytes

Initial experiments examined the binding of the anti-TfR MoAbs to human lymphocytes. From previous reports of others, it is known that resting human lymphocytes express far fewer transferrin-binding sites than lymphocytes that have been activated by mitogens.<sup>15</sup> Indirect immunofluorescence was used to identify binding to TfR on human lymphocytes (Table 1). Only 6%–8% of freshly obtained peripheral blood mononuclear cells bound B3/25 or 42/6 anti-TfR

MoAb, while 72% bound a T-cell-specific MoAb (T101) and 83% bound MoAb (T29/33) to an irrelevant glycoprotein (T200). After 24 and 96 hr of incubation in the presence of PHA, the percent of cells binding the two anti-TfR MoAbs increased markedly, while the binding of the two control MoAbs (T101 and T29/33) changed only slightly.

The data from this experiment give an estimate of changes in the relative number of transferrin receptor sites per cell during culture. Inspection of the cytofluorometry patterns showing binding of MoAb demonstrated a broad, heterogeneous spread of FITC intensity for the PHA-stimulated lymphocytes, without a clear modal peak (Fig. 1). When the mean fluorescence intensities of stimulated and resting lymphocytes were compared, the data showed a twofold rise after 24 hr of stimulation, when some cells are just beginning to enter S phase, and a sevenfold increase at 96 hr, when the culture is at near maximal proliferation (Table 1).

### Effects of Anti-TfR MoAb on Lymphocyte Proliferation

Addition of anti-TfR MoAb 42/6 to human lymphocyte cultures stimulated with PHA resulted in a concentration-dependent reduction in cell proliferation (Fig. 2A). At concentrations of 5 and 50  $\mu\text{g}/\text{ml}$ , the mitogenic response was markedly depressed. In contrast, exposure to anti-TfR MoAb B3/25 resulted in only a modest decrease in cell number (Fig. 2B), which was not concentration dependent. Comparable results were obtained when the proliferative response was assayed by incorporation of <sup>3</sup>H-thymidine (Fig. 3). DNA synthesis peaked ahead of the maximal cell number observed, as would be expected. Further control data were obtained with MoAb T29/33 to an unrelated surface glycoprotein and MoAb T101 to a T-cell-specific surface antigen. Exposure to each of these antibodies resulted in a modest (20%) decrease in proliferation, which was not concentration dependent. The data are comparable to the observations in Figs. 2B and 3B and are not shown.

When 42/6 anti-TfR MoAb was added to cultures 48 hr after addition of PHA, the proliferative response also was suppressed, although fewer lymphocytes died during the culture period (Fig. 4). This suggests that access to transferrin is required throughout the culture period and is not involved merely with an initiating event. Further studies assessing cell cycle kinetics in these lymphocyte cultures confirmed this conclusion.

### Cell Cycle Kinetics With MoAb

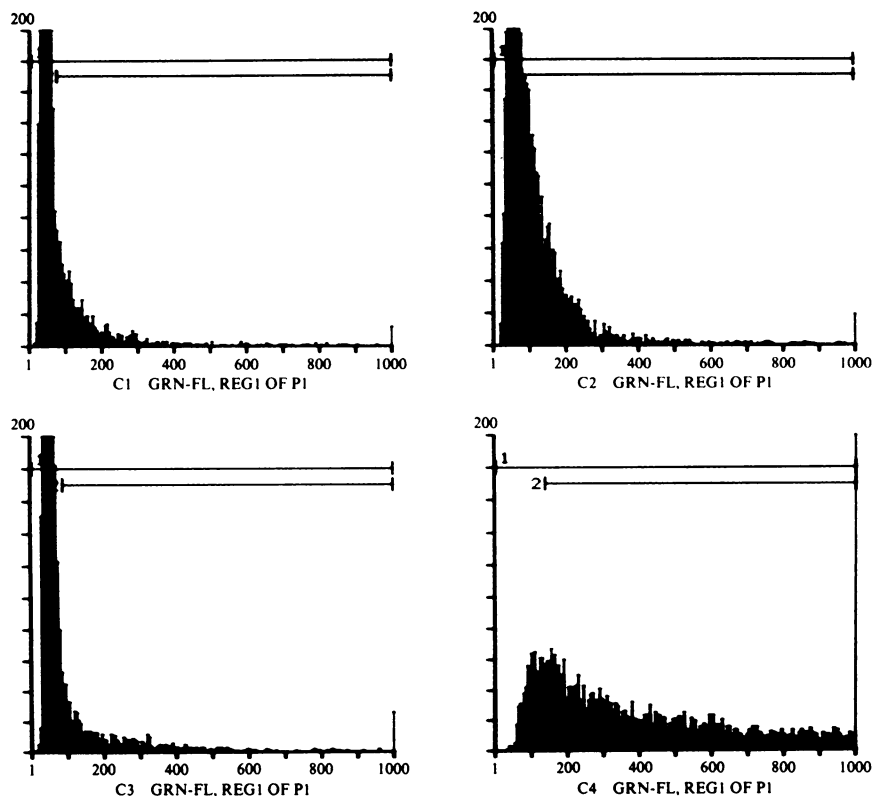
The effects of MoAb on cell cycle phase distributions were determined by cytofluorometry (Fig. 5).

**Table 1. Binding of MoAb to Lymphocytes in PHA-Stimulated Culture\***

Hours of Culture	MoAb			
	42/6	B3/25	T101	T29
Cells fluorescing (%)				
0	6	8	72	83
24	45	56	78	88
96	70	70	—	86
Increase in fluorescence (compared to Day 0)				
24	1.7	2.0	1.0	1.0
96	6.9	7.6	1.0	1.0

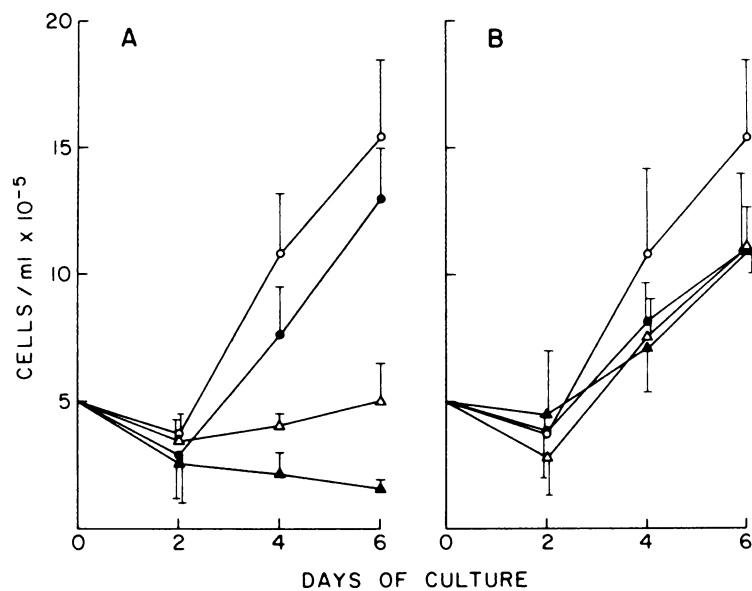
\*The data in the upper part record percent of cells with fluorescence greater than that generated in the control samples, reacted with irrelevant antibody (Materials and Methods). The data in the lower part are the ratio of mean cell fluorescence after varying periods of incubation compared with mean cell fluorescence at the start of the culture.

**Fig. 1.** Tfr site content of cultured human lymphocytes. Cells were stained with anti-Tfr MoAb 42/6 and with FITC-labeled goat anti-mouse IgG, and fluorescence was assayed by cytofluorometry, as described in Materials and Methods. Upper left panel: 1-day cultures, no mitogen. Upper right panel: 1-day cultures, PHA 1  $\mu\text{g}/\text{ml}$ . Lower left panel: 4-day cultures, no mitogen. Lower right panel: 4-day cultures, PHA 1  $\mu\text{g}/\text{ml}$ . Ordinate: cell number; abscissa: green fluorescence, presented as relative intensity over 1,000 channels. The term Reg of P1 in the legend for the abscissa indicates that viable single lymphocytes are being analyzed (see Materials and Methods). The entire fluorescence profile is presented, without subtracting nonspecific fluorescence detected with the control MoAb.



Freshly obtained lymphocytes contained 97%  $G_1$  ( $G_0$  and  $G_1$ ), cells, as expected, and the normal increase in S and  $G_2 + M$  phase cells occurred between 24 and 48 hr after culture with PHA was initiated. When 42/6 MoAb, 50  $\mu\text{g}/\text{ml}$ , was added at the time of culture initiation, the percent of cells entering S phase increased comparable to control cultures during the first 48 hr, and then fell to low levels during the subsequent period of culture. Examination of the flow

cytofluorometry DNA histogram patterns demonstrated that the MoAb-treated cells were blocked early in S phase. Entry into  $G_2$  was practically nil during the entire experiment. The percent of cells in  $G_1$  declined during the first 48 hr, when cells were entering S phase, and then increased to 90% while cells in S phase declined. Since it was shown that the viable cell count fell dramatically under these conditions (Fig. 2), the cytofluorometry data suggest that, early in the culture,



**Fig. 2.** Proliferation of PHA-stimulated lymphocytes exposed to varying concentrations of anti-Tfr MoAb 42/6 (A) and B3/25 (B). Viable cell counts were obtained by the Trypan blue dye exclusion method. Data are means  $\pm$  SEM of 3 separate experiments. (○) No MoAb; (●) 0.5  $\mu\text{g}/\text{ml}$ ; (Δ) 5  $\mu\text{g}/\text{ml}$ ; (▲) 50  $\mu\text{g}/\text{ml}$ .

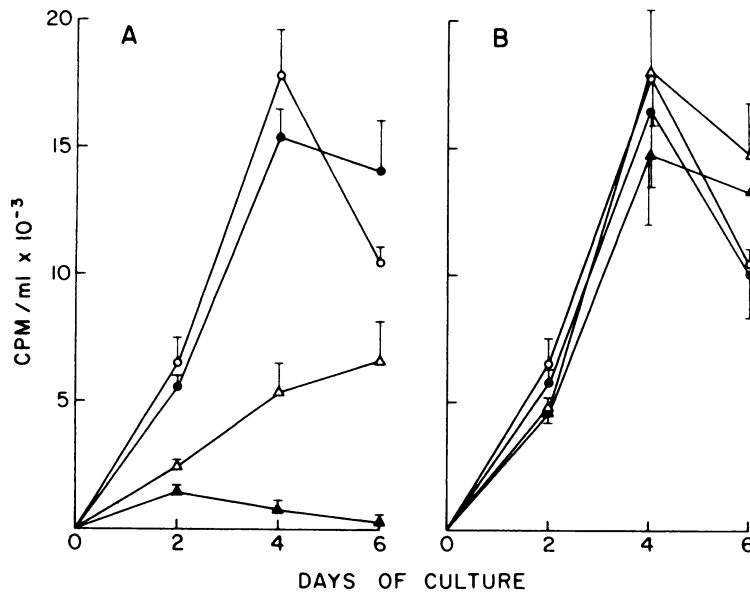


Fig. 3. DNA synthesis in PHA-stimulated lymphocytes estimated by incorporation of  $^3\text{H}$ -thymidine. Cultures identical to Fig. 1 were exposed to anti-TfR MoAb 42/6 (A) and B3/25 (B). (O) No MoAb; (●) 0.5  $\mu\text{g}/\text{ml}$ ; ( $\Delta$ ) 5  $\mu\text{g}/\text{ml}$ ; ( $\blacktriangle$ ) 50  $\mu\text{g}/\text{ml}$ .

cells enter S phase and arrest (without progression to  $G_2$ ) and then die (resulting in decreased percent S cells); later in the culture, cells also die in  $G_1$  phase.

When 42/6 MoAb, 50  $\mu\text{g}/\text{ml}$ , was added 48 hr after PHA stimulation, the distribution of lymphocytes in the phases of the cell cycle was similar to control cultures not exposed to MoAb, in spite of the fact that cell proliferation was inhibited by the late addition of MoAb. Cell counts did not rise appreciably between days 2 and 6 (Fig. 4). The data suggest that actively proliferating lymphocytes are sensitive to inhibition by 42/6 MoAb during all phases of the cell cycle and confirm the conclusions reached with the experiment

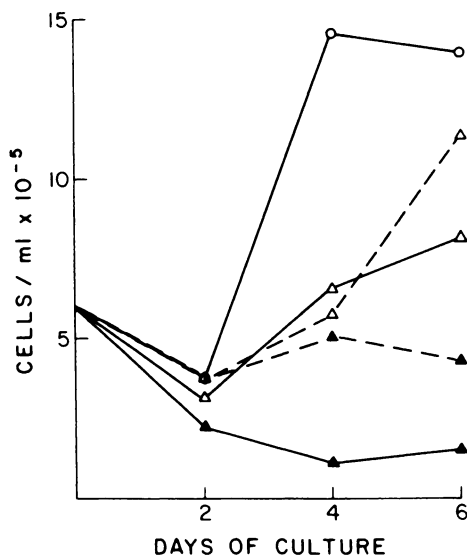


Fig. 4. Proliferation of PHA-stimulated lymphocytes exposed to anti-TfR MoAb 42/6 either at initiation of culture (—) or after 2 days of culture (----). Viable cell counts are plotted: (O) No MoAb; ( $\Delta$ ) 5  $\mu\text{g}/\text{ml}$ ; ( $\blacktriangle$ ) 50  $\mu\text{g}/\text{ml}$ .

in Fig. 4 that transferrin is required for maintenance of lymphocyte proliferation responses.

#### Reversal of MoAb Antiproliferative Effect

The reversibility of the MoAb-induced block in proliferation also was examined. Figure 6 shows a series of experiments in which PHA-stimulated cultures were incubated with 42/6 MoAb for periods of between 0 and 72 hr, after which interval, the MoAb

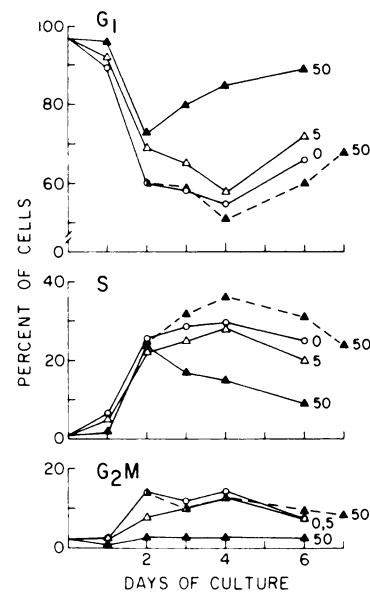


Fig. 5. Cell cycle kinetics in the presence of anti-TfR MoAb 42/6. The data pertain to aliquots of the cells from the experiment presented in Fig. 4, with MoAb added at the initiation of culture (—) or after 2 days of culture (----). Cell cycle distributions were determined by cytofluorometry. The percentages of cells in the various phases of the cell cycle are plotted: (O) no MoAb; ( $\Delta$ ) 5  $\mu\text{g}/\text{ml}$ ; ( $\blacktriangle$ ) 50  $\mu\text{g}/\text{ml}$ .

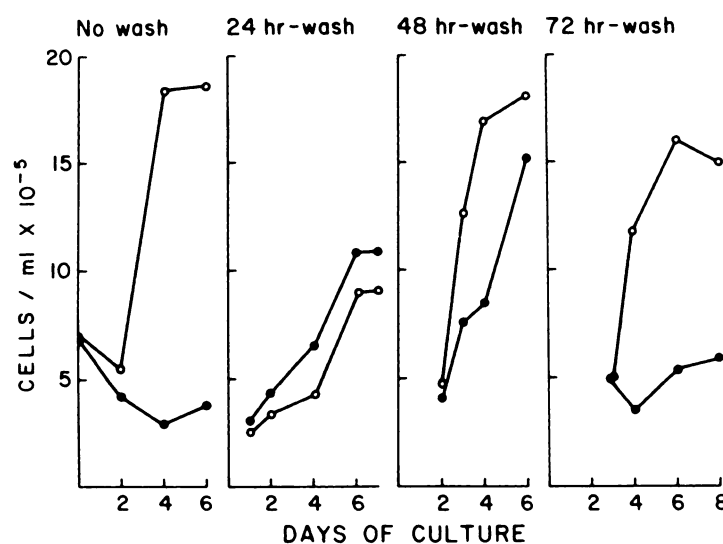


Fig. 6. Reversibility of inhibition by anti-TfR MoAb 42/6. PHA-stimulated lymphocytes were cultured in the presence (●) or absence (○) of MoAb for varying periods of time, after which both control and MoAb-treated cells were washed by centrifugation, resuspended in fresh medium at comparable cell concentrations, and cultured further in the absence of MoAb. Viable cell counts are plotted. The dip in cell counts during the first 24 hr of culture is seen typically and can be attributed to cell death<sup>16</sup> plus some PHA-induced clumping, which decreases later in the culture period.

was removed by a centrifugational washing step, and the cells were cultured for 3–4 additional days. Removal of MoAb after 24 hr of exposure resulted in reversal of the antiproliferative effect, with cell counts rising nearly identically to those in cultures not exposed to MoAb. The recovery also was nearly complete after 48 hr, but did not occur when cells were exposed to antibody for 72 hr before washing. Thus, PHA-stimulated lymphocytes are able to recover from transferrin deprivation for periods up to 48 hr.

#### Reversibility of MoAb Effect With Iron

To determine whether inhibitory effects of 42/6 MoAb on lymphocyte proliferation could be prevented by providing an alternative source of soluble iron, various amounts of ferric nitrilotriacetic acid<sup>8</sup> were added to cultures (Fig. 7). In the presence of 50  $\mu\text{g}/\text{ml}$  anti-TfR MoAb 42/6, addition of ferric nitrilotriacetic acid resulted in a partial reversal of the inhibitory effect of 42/6 MoAb on cell proliferation. However, even at the highest concentration tested (200  $\mu\text{M}$ ), the number of viable cells found in antibody-treated cultures was less than in the untreated cultures.

#### DISCUSSION

PHA-stimulated human lymphocytes are well suited for examining the antiproliferative effects of MoAbs, because the proliferation kinetics of these "resting phase" cells in response to this mitogen have been characterized thoroughly. In mitogen-activated cultures, normal human lymphocytes in a  $G_0$  (or early  $G_1$ ) state are stimulated to enter the cell cycle randomly over a period of 72 hr.<sup>16</sup> Concurrent with entry into the proliferative cycle, human lymphocytes express increased numbers of TfR sites.<sup>15</sup> The cytofluorometry data (Fig. 1) indicate that there is a broad

distribution in the number of TfR sites per lymphocyte. Similar patterns have been observed in studies with MoAb OKT9, which also binds to the TfR.<sup>17</sup>

The present experiments demonstrate clearly that anti-TfR MoAb 42/6 added to lymphocyte cultures at the initiation of mitogen stimulation interferes with the capacity of the cells to traverse S phase of the cell cycle. The inhibition of cell cycle traversal is reversible when MoAb is removed from the culture after up to 48 hr. Inhibition can be induced by adding 42/6 MoAb 48 hr after initiation of the mitogenic response, indicating that transferrin is required continually for the proliferation of normal lymphocytes in mitogen-driven cultures. It appears that, once T lymphocytes are actively

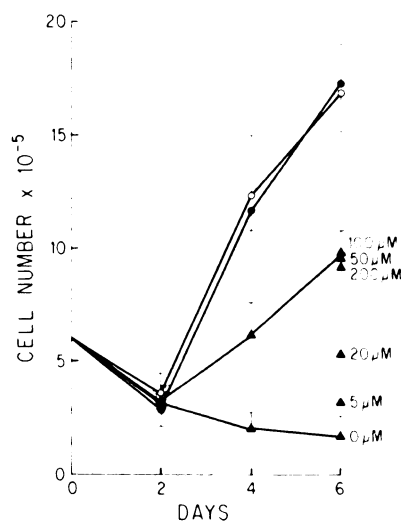


Fig. 7. Reversal of MoAb-induced growth inhibition by addition of iron. The two control cultures were unexposed to MoAb and were grown in the absence (○) or presence (●) of 100  $\mu\text{M}$  iron. Experimental cultures (▲) were exposed to 50  $\mu\text{g}/\text{ml}$  42/6 MoAb plus varying concentrations of ferric nitrilotriacetic acid (5–200  $\mu\text{M}$ ), as stated on the figure. Data presented on the linear plots represent the mean  $\pm$  SEM of 3 experiments.

proliferating, they are sensitive to the inhibitory effects of 42/6 MoAb throughout all phases of the cell cycle.

As with the CCRF-CEM T-leukemia cell line studied previously,<sup>4</sup> addition of Fe-transferrin did not reverse the blocking effect of 42/6 MoAb (data not shown). However, in contrast to the results with the T-leukemia cell line, inhibition of the proliferative response of normal T lymphocytes by the anti-TfR MoAb was partially reversed by the addition of iron in the form of ferric nitrilotriacetic acid. The reason for this difference is not clear, but it suggests that the iron requirements of normal T lymphocytes can be satisfied to some extent by nontransferrin-bound iron. This result also provides further support for the view that 42/6 MoAb is inhibiting lymphocyte proliferation by depriving the cells of essential iron. However, it is possible that the MoAb is blocking growth by another mechanism in addition to iron deprivation.

The experiments with B3/25 MoAb, which binds to the TfR but does not block access of transferrin to the receptor, add evidence that inhibition of proliferation by MoAb was not due simply to a nonspecific effect resulting from binding to the receptor molecule. It must be pointed out that 42/6 MoAb has an IgA

isotype, unlike B3/25 and the other control MoAbs used in these studies, which are all IgGs. Unfortunately, we do not have an IgA MoAb that can bind to lymphocyte surface membranes at another site and serve as an isotype control.

It is clear that, initially, inhibition of normal lymphocyte proliferation is a static rather than a tidal event, which is reversible following removal of MoAb for up to 48 hr. It is possible that reversibility may not be achievable as readily in the case of malignant cells exposed to antibody. The literature provides many examples of situations where transformed malignant cells do not adapt well to deprivation from growth factors or other nutrients required for proliferation, in contrast with normal cells, which become quiescent and maintain viability.<sup>18</sup> Future experiments will examine this point carefully, since such selectivity might have implications for the therapy of malignant disease.

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