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TRANSFERRIN RECEPTORS: STRUCTURE AND FUNCTION

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The advent of monoclonal antibodies and improved molecular biologic techniques have had a tremendous impact upon modern biology. Nowhere is this more apparent than in studies of the cell surface. Until recently, only rudimentary information about the structure of most cell surface molecules had been obtained and little progress had been made in understanding how they function. Now, however, as a result of these new technologies, rapid progress is being made in both areas. Here, we will review the current status of transferrin receptors and then focus on two particular aspects of our own ongoing work that both involve the use of monoclonal antibodies against transferrin receptors. About two years ago, we obtained monoclonal antibodies against transferrin receptors of both human and murine cells that block receptor function. The effects of these antibodies on cell growth *in vitro* and *in vivo* will be described and the broader implications of this work for other functional cell surface molecules will be discussed. The other topic concerns the selection by fluorescence-activated cell sorting of mouse L cells that express the human transferrin receptor on their surface after transformation with total human DNA. These L cell transformants have interesting properties and provide an approach to cloning the human transferrin receptor gene. This methodology may have general applicability for the isolation of the genes of other cell surface molecules against which monoclonal antibodies have been obtained.

BACKGROUND TO TRANSFERRIN RECEPTOR STUDIES

Iron plays an important role in cell growth catalyzing key reactions in energy metabolism and DNA synthesis. Under most physiological conditions, iron exists in its oxidized ferric state, and at neutral pH, ferric salts are hydrolyzed to insoluble ferric hydroxide. To overcome this problem, organisms have developed a variety of binding systems to sequester iron in a soluble form and transport it into the cell. The cellular iron transport system used by vertebrates consists of the serum iron-binding protein, transferrin, that specifically interacts with a cell surface receptor which then facilitates transport across the cell membrane. Although the existence of specific receptors for transferrin was first proposed by Jandl and Katz in 1963 [1], the transferrin receptor was not purified and adequately characterized until 1979 [2, 3]. Much of the early work on transferrin receptors was focussed upon the maturing cells of the erythroid lineage which have a high iron requirement for heme synthesis and the placental trophoblast which channels iron to the developing embryo from the maternal circulation. However, at about the same time that transferrin receptors were isolated from human placenta, it was recognized that many other cell types also express transferrin receptors and that proliferating cells express much larger numbers of receptors than resting cells [4–6].

The association of transferrin receptor expression and cell growth was confirmed as a result of an independent line of investigation. Several groups obtained monoclonal antibodies against a major human cell surface glycoprotein that was shown to be selectively expressed on proliferating cells [7–9]. The cell surface glycoprotein was later shown to be the transferrin receptor in experiments demonstrating that the monoclonal antibodies could precipitate transferrin in association with the glycoprotein [10–12]. The initial work on transferrin receptors detected either by transferrin binding or with monoclonal antibodies has provided the basis for a variety of new studies. For example, the recycling of transferrin receptors is currently under intensive investigation and provides an excellent model for the study of plasma membrane receptor traffic to and from the cell surface [13–18]. A different question is the coordinate genetic regulation of transferrin receptors with cell growth which represents an interesting and unusual problem in gene regulation. Finally, as a consequence of their association with proliferating cells, transferrin receptors are also of interest in relationship to malignancy. It has been shown that some tumor tissues selectively express transferrin receptors relative to most normal tissues [19] and this selectivity may eventually be of some value diagnostically or in the treatment of cancer [20, 21].

STRUCTURE OF TRANSFERRIN RECEPTORS

The general structural features of the transferrin receptor of human cells are illustrated schematically in Fig. 1. The molecule is a disulphide-bonded dimer consisting of two identical 95,000 molecular weight subunits. It is a transmembrane glycoprotein that contains at least three N-asparagine linked oligosaccharides. Both high mannose and complex-type oligosaccharides are found on the mature glycoprotein [22–24] and the bulk of the transferrin receptor is exposed on the cell surface. The molecule can be cleaved on the cell surface with a low concentration of trypsin to generate a soluble fragment of

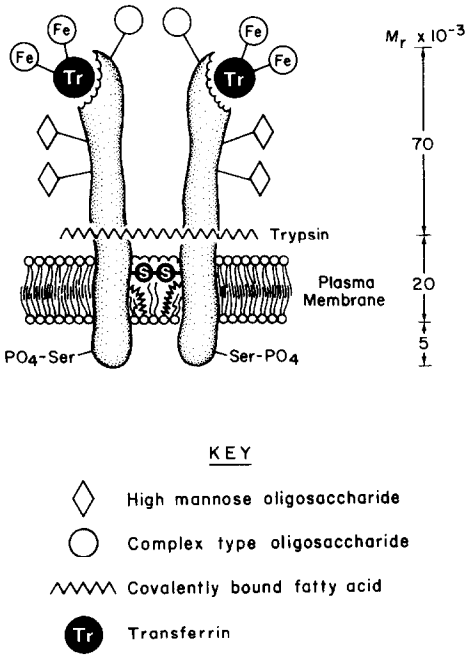

Fig. 1. Schematic representation of the cell surface transferrin receptor. It should be noted that the precise sites of fatty acid, phosphoserine and disulphide bonds have not been completely localized. Their likely positions in the tryptic fragment associated with the cell membrane are indicated and have been selected by analogy with what is known about other transmembrane glycoproteins. Also, the orientation of transferrin receptor in the membrane and the number of times the receptor polypeptide spans the membrane is unknown.

70,000 apparent molecular weight which is still able to bind transferrin leaving the region of the molecule containing the disulfide bond embedded in the membrane [14, 22, 24]. The portion of the molecule exposed on the cytoplasmic face of the plasma membrane has been estimated to have an apparent molecular weight of approximately 5000 and, at least in some cell types, serine residues located in this region are phosphorylated [24]. As shown by Omary and Trowbridge [22, 23], the transferrin receptor is also modified post-translationally by the addition of fatty acid residues. Each subunit of the dimeric transferrin receptor contains a binding site for transferrin [24]. Most estimates of the association constant of transferrin binding fall in the range of $2\text{–}7 \times 10^{-9}$ M [3, 4, 13, 25, 26]. Evidence from immunological and peptide mapping studies [27, 28] suggests that transferrin receptors of different cell types are closely related if not identical. However, a monoclonal antibody has been identified that appears to preferentially react with transferrin receptors of human erythroid precursors [29], and the question of whether there is more than one type of transferrin receptor remains open. One point of interest is that the human transferrin receptor is apparently polymorphic [30].

The biosynthesis of transferrin receptors follows the metabolic pathways that have been described previously for other cell surface glycoproteins [31]. There is evidence that the acylation of the transferrin receptor can occur more than two days after the synthesis of the receptor polypeptide suggesting the receptor may be acylated and deacylated during its lifetime. Although the turnover of the lipid moiety appears to be faster than the transferrin receptor molecule itself [23], an acylation–deacylation cycle is unlikely to be essential for receptor recycling. The significance of covalently bound lipid is unknown, although several general suggestions have been put forward [32, 33]. As with the viral membrane glycoproteins that have been isolated, the region of the transferrin receptor which contains the covalently-bound fatty acid is located close to the lipid bilayer of the membrane and one suggestion is that the lipid may play some role in anchoring the receptor into the membrane. Although the structural features of the transferrin receptor just described have been mainly obtained from studies of the human receptor, transferrin receptors of other species [34] appear to be similar.

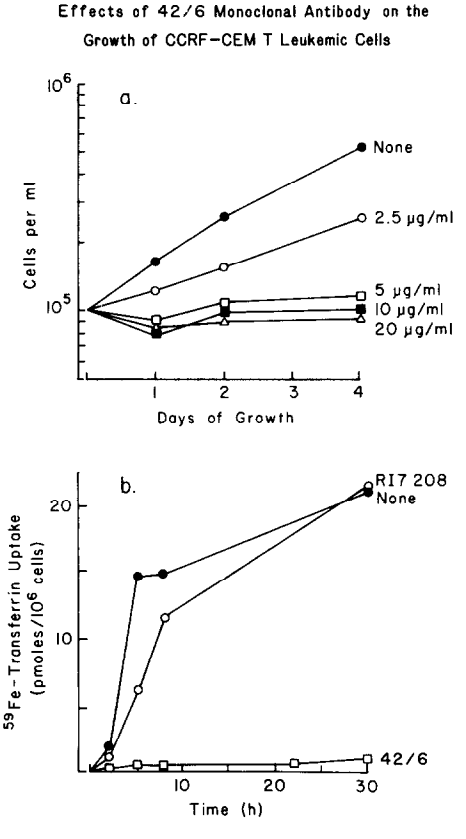

Fig. 2. Effects of the murine anti-human transferrin receptor monoclonal antibody, 42/6, on iron uptake and growth of CCRF-CEM cells. (A) shows the inhibition of growth of the human T leukemic cell line, CCRF-CEM, by 42/6 monoclonal antibody. Experimental details are given in Ref. [35]. (B) shows the specific inhibition by 42/6 monoclonal antibody of transferrin-mediated iron uptake by CCRF-CEM cells. Experimental details of the assay are described in Ref. [34].

MONOCLONAL ANTIBODIES THAT BLOCK RECEPTOR FUNCTION

The monoclonal antibodies originally obtained against the transferrin receptor of human cells did not interfere significantly with receptor function. However, we were recently able to select an antibody, designated 42/6, that blocked transferrin binding to the human transferrin receptor [15]. As shown in Fig. 2, this antibody was not only capable of blocking transferrin-mediated iron uptake in a human T leukemic cell line, CCRF-CEM, but it also inhibited cell growth at low concentrations. As little as 2.5 μg/ml of purified 42/6 monoclonal antibody was sufficient to have a detectable effect on the growth of CCRF-CEM leukemic cells and growth was totally inhibited at 5–10 μg/ml of antibody. Measurement of the DNA content of cells whose growth have been inhibited by 42/6 monoclonal antibody showed that there was progressive accumulation of cells in S-phase of cell cycle [35]. This suggests that at least for CCRF-CEM cells the major effect of iron deprivation is on some metabolic process associated with DNA synthesis. One candidate for this is the enzyme ribonucleotide reductase which plays a key regulatory role in DNA synthesis and requires iron for the formation of an organic free radical necessary for the catalytic reduction of ribonucleotides [36]. It is interesting in this regard that the effects on cell growth of the chelating agent, picolinic acid, which inhibits the incorporation of iron into cells and hydroxyurea, which inhibits ribonucleotide reductase, are similar to that of the anti-transferrin receptor antibody [37]. However, for reasons that are presently obscure, not all cell types are sensitive to the growth inhibitory effects of the anti-transferrin receptor antibodies, even though transferrin-mediated iron uptake into resistant cells is inhibited to approximately the same extent as that in sensitive cells [38]. Further work is required to understand the regulation of iron uptake and storage in different kinds of cells before this problem can be resolved.

It seemed possible, given the fact that tumor tissues often express high numbers of transferrin receptors, that if monoclonal antibodies that block transferrin receptor function *in vitro* also inhibit growth *in vivo*, then they may be potentially useful as therapeutic agents in the treatment of cancer. In order to investigate this idea, we sought to develop an animal model system to study the effects of anti-transferrin receptor antibodies on tumor cell growth *in vivo*. The first step was to try to find a rat monoclonal antibody that reacted with the murine transferrin receptor. An antibody, designated R17 208, was identified and shown to have growth inhibitory properties similar to monoclonal antibody 42/6 [34]. For our initial experiments, we chose to study the transplantable AKR mouse T-cell leukemia, SL-2. This cell line has previously been used by Bernstein and his colleagues to investigate the requirements for effective serotherapy in anti-Thy-1.1 monoclonal antibodies [39, 40]. These earlier studies provided a standard against which to compare the anti-tumor activity of anti-transferrin receptor antibodies. The model was also attractive in that if therapeutic effects are obtained with the transplantable T-cell leukemia, studies could then be extended to include spontaneous T-cell leukemias in AKR mice. Furthermore, it is known that T-cell leukemias in man frequently express high numbers of transferrin receptors [7, 10, 11, 41] and the *in vitro* growth of human T-leukemic cell lines are particulary sensitive to the effects of monoclonal antibody 42/6 [35]. Consequently T-cell leukemias might be expected to be a favorable starting point for attempting immunotherapy with anti-transferrin receptor antibodies. The results of one of several trials of immunotherapy with the purified anti-mouse transferrin receptor antibody, R17 208, which produced similar results are shown in Fig. 3 and 4.

AKR/J mice were each inoculated subcutaneously with 1×10^6 SL-2 T-leukemic cells [a dose 100-fold greater than the 50% lethal dose (LD_{50})] [39]. Groups of six mice were then either left untreated or given purified anti-transferrin receptor antibody R17 208 or anti-Thy 1.1 monoclonal antibody 19E12 intravenously on day 0 and interperitoneally on day 4 and day 7 at a dose of 3 mg per injection. As expected from the previous work of Bernstein and his colleagues, treatment of the tumor-bearing mice with the monoclonal anti-Thy 1.1 antibody 19E12, significantly prolonged their survival (Fig. 3). However, as shown in Fig. 4, the anti-Thy 1.1 monoclonal antibody had no effect on the growth of the primary subcutaneous tumor. This is also in agreement with previous work in that the increased survival of the anti-Thy-1 antibody-treated mice is due to the effects on metastatic disease [42]. The effects of treatment with the anti-transferrin receptor antibody were significantly different. Not only was the survival of the tumor-bearing mice considerably prolonged (a mean survival time of R17 208 antibody-treated mice of 38.1 ± 3.7 days versus a mean survival time of 22.0 ± 3.6 days for the control group) but there was a very marked effect upon the growth of the primary

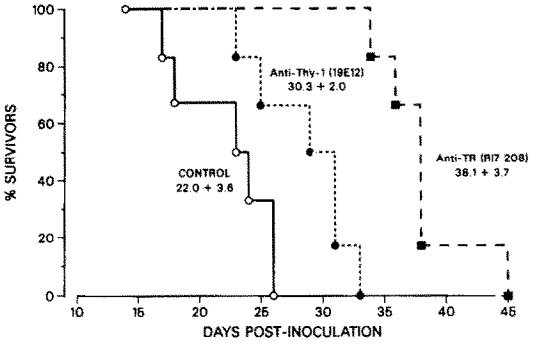

Fig. 3. Treatment of AKR mice with rat anti-murine transferrin receptor antibody, R17 208, prolongs survival of mice challenged with SL-2 leukemia cells. A group of six-week old female AKR mice were inoculated with 1×10^6 SL-2 cells at subcutaneous sites on the back. Three milligrams of purified R17 208 monoclonal antibody (anti-murine transferrin receptor) or monoclonal antibody 19E12 (anti-Thy-1.1) were given on days 0, 4 and 7. The first injection was intravenous, the succeeding two doses of antibody were intraperitoneal. The figures shown are the mean survival time of mice in each group ± 1 standard error.

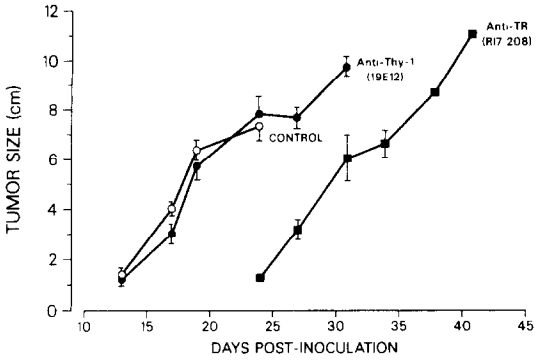

Fig. 4. The effects of anti-transferrin receptor antibody, R17 208, on the growth of SL-2 cells at the primary tumor site in AKR/J mice. The data shown are from the same experiment as shown in Fig. 3. Mice were inoculated with SL-2 leukemia cells and given antibodies as described in Fig. 3. The figure shows the mean tumor size on each day measured as the average of 2 perpendicular axis with calipers in each group of mice.

tumor (Figs. 3 and 4). As shown in Fig. 4, there was a delay of approximately 10 days between the appearance of tumors in the control group of mice and the detection of palpable tumors in the anti-transferrin receptor antibody-treated mice; however, once tumors appeared in the antibody-treated mice they grew at the same rate as those of the control group. In fact, from these results, given that the doubling time of SL-2 cells is on the order of 16 hrs, it would appear that R17 208 monoclonal antibody reduced the initial tumor inoculum by approximately 5 orders of magnitude. The questions that are now under investigation using this model system are: (1) Can more effective immunotherapy be obtained using a combination of monoclonal antibodies such as 19E12 and R17 208 which produced their effects by different mechanisms? (2) Can more prolonged treatment with the anti-transferrin receptor monoclonal antibody induce cures in tumor-bearing mice? (3) What are the effects of immunotherapy with monoclonal antibody R17 208 on established SL-2 tumors? (4) Is it possible to prove that the *in vivo* effects of R17 208 monoclonal antibody are the consequence of iron deprivation as indicated by the results of *in vitro* experiments? This last question can be answered rather elegantly as mutant cell lines have been derived from cultured mouse T-cell leukemic cell lines that are no longer sensitive to the growth inhibitory effects of R17 208 monoclonal antibody *in vitro* (J. Lesley and R. Schulte, Salk Institute, unpublished results). It would be predicted that if R17 208 monoclonal antibody was inhibiting the *in vivo* growth of tumors by the same mechanism as *in vitro*, that whereas the parental leukemic cell lines would be sensitive to antibody treatment *in vivo*, the mutant cell lines would not be. It remains to be seen whether this proves to be the case.

Another important aspect of these model immunotherapy studies with the anti-transferrin receptor antibody is that despite the fact that some cells in normal tissues express transferrin receptors, so far there has been no evidence of toxicity in the treated mice. There are many reasons why some tumors may prove to be more sensitive to the effect of anti-transferrin receptor antibodies that block iron uptake in most normal tissues. These include elevated levels of transferrin receptors on particular tumors relative to normal dividing cells and the possibility that consequences of iron deprivation to different cell types may be different.

The results just described suggest that the idea of using monoclonal antibodies to block the function of essential growth-related receptors as a viable approach to the treatment of malignancy seems worth further investigation. Although we are concerned here with transferrin receptors, the approach is generally applicable and monoclonal antibodies have been obtained against epidermal growth factor receptor [43], insulin receptor [44, 45] and the interleukin 2 (IL-2) receptor of human cells [46]. It has been shown that the monoclonal antibody against the IL-2 receptor blocks IL-2 binding and inhibits the *in vitro* growth of human T lymphocytes [46]. This example provides further evidence that mono-

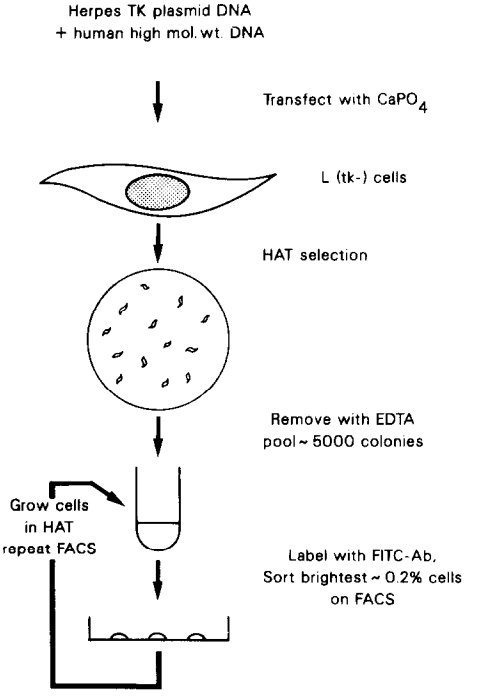

Fig. 5. Schematic representation of the cotransformation protocol used to obtain mouse L cell transformants expressing the human transferrin receptor. Mouse L cells were cotransformed with a calcium phosphate precipitate containing both the Herpes simplex thymidine kinase gene and high molecular weight DNA from the human T leukemia cell line, CCRF-CEM. L cell colonies containing the thymidine kinase gene were selected by growth in selective HAT medium. The colonies were grown up and pooled, then stained with monoclonal antibodies against the human transferrin receptor and a FITC-rabbit anti-mouse IgG second-stage antibody. The stained cells were then passed through the fluorescence-activated cell sorter and approximately 0.2% of the most fluorescent cells collected. These cells were grown up and again stained and sorted for expression of the human transferrin receptor. Additional experimental details can be found in Ref. [52].

clonal antibodies that block essential biological functions can be used to regulate cell growth.

SELECTION AND PROPERTIES OF MOUSE L CELL TRANSFORMANTS EXPRESSING THE HUMAN TRANSFERRIN RECEPTOR

It is likely that much of the progress to be made in the next few years in understanding the structure and function of membrane molecules will come from the application of molecular biologic techniques. In the case of the transferrin receptor, cloning the gene would not only contribute to an understanding of its primary structure, but also provide information relevant to several interesting genetic questions. Recent chromosomal analysis has shown that the structural genes for the transferrin receptor, probably transferrin itself, and P97, a melanoma tumor-associated cell surface antigen that exhibits primary sequence homology with transferrin and binds Fe^{3+}, each map in man to chromosome 3 [47–50]. On this basis, it has been suggested that there may be a region within chromosome 3 containing genes involved in iron transport and that in some circumstances rearrangement in this region may be associated with malignant transformation [50]. This question, together with the problem of how the expression of transferrin receptors is coordinately regulated with cell proliferation and the continuing debate as to whether all cell types express structurally identical transferrin receptors require a study at the molecular biologic level. As an approach to investigating these problems, we have obtained mouse L-cell transformants expressing the human transferrin receptor. The protocol we employed is shown schematically in Fig. 5. L (tk^-) cells were cotransformed with total high molecular weight DNA from a human leukemic cell line, CCRF-CEM, and a plasmid containing the Herpes simplex thymidine kinase (tk) gene using the calcium phosphate method [51]. Transformants containing the tk gene were selected by growth in Dulbecco's modified Eagles medium containing 10% horse serum and hypoxanthine/aminopterin/thymidine (HAT) medium. Resistant colonies were then pooled, stained with a mixture of mouse monoclonal antibodies against the human transferrin receptor, followed by fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin and then sorted by flow cytometry. The brightest 1–2% of the viable fluorescent cell population was collected, grown up and resorted. Each independent trial yielded approximately 2–4 × 10^3 HAT-resistant colonies and from a total of 30,000 such colonies, two independent L-cell transformants have been obtained that express the human transferrin receptor on their cell surface [52]. One of these transformants, J4, has been characterized extensively. As shown in Fig. 6, after three cycles of sorting, a cell population was obtained that was more than 90% positive when stained with the B3/25 monoclonal antibody against the human transferrin receptor [52]. This transformant was also positive when stained with a panel of other anti-human transferrin receptor monoclonal antibodies. The level of expression of the murine transferrin receptor on J4 cells was comparable to that of L(tk^-) cells and karyotypic analysis confirmed that the transformant was derived from the original L-cell population. The human and mouse transferrin receptors were expressed in approximately equal amounts on the J4 transformant (Fig. 6).

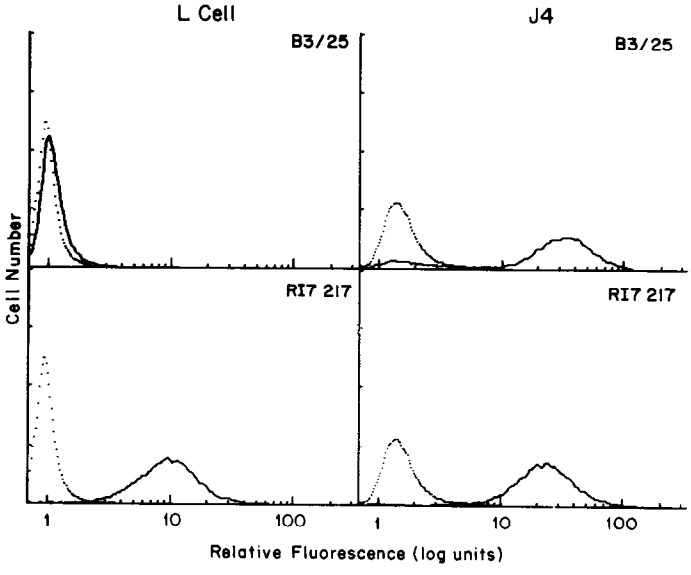

Fig. 6. Expression of the human transferrin receptor on mouse L cells after transformation with human DNA and fluorescence-activated cell sorting. The figure shows the fluorescence-activated cell analysis of the J4 L cell transformant and L cells stained with either monoclonal antibody B3/25 against the human transferrin receptor or monoclonal antibody R17 217 against the murine transferrin receptor. The solid lines represent specific staining and the dotted lines represent control staining in which the first-stage monoclonal antibody was omitted. All the results have been expressed relative to the mean fluorescence of L cells stained with the second-stage fluorescent antibody only. Further experimental details can be found in Ref. [52].

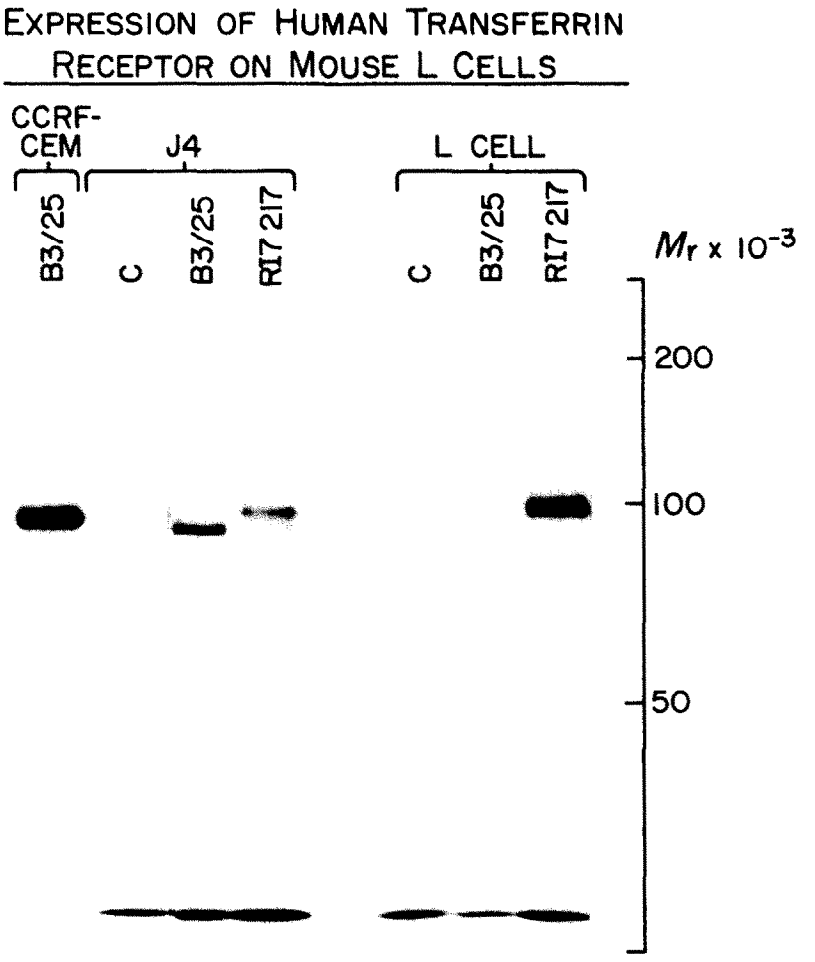

Fig. 7. SDS-polyacrylamide gel analysis of murine and human transferrin receptors on the J4 L cell transformants. Cells were iodinated by the lactoperoxidase technique and immunoprecipitates prepared with monoclonal antibodies B3/25 and R17 217. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. The track marked C represents control immunoprecipitates in which the first-stage monoclonal antibody was omitted. Further experimental details are given in Ref. [52].

SDS-polyacrylamide gel analysis of immunoprecipitates prepared from detergent lysates of J4 cells labeled by lactoperoxidase catalyzed iodination are shown in Fig. 7. The results confirm that the transformed cells express both murine and human transferrin receptors as shown by the precipitation of labeled species of about 90–100 K under reducing conditions with both B3/25 monoclonal antibody and R17 217, a rat monoclonal antibody against the murine transferrin receptor (J. Lesley and R. Schulte, unpublished results). The murine and human transferrin receptors can be distinguished on the basis of their apparent molecular weights on SDS-PAGE, the mouse transferrin receptor migrating significantly slower than the human receptor. The receptor from both species migrated as dimers of approximately 190 K under nonreducing conditions. Two further points of interest emerged from SDS-PAGE analysis. First, the human transferrin receptor precipitated by B3/25 from J4 cells is of lower apparent molecular weight than that from CCRF-CEM cells. Second, in addition to the major species precipitated by B3/25 and R17 217 monoclonal antibodies, each antibody (most clearly seen in the case of R17 217 in the experiments illustrated in Fig. 7) precipitates a minor band. These minor components are not observed when mouse and human transferrin receptors are isolated from L cells or CCRF-CEM cells, respectively and almost certainly arises as the result of the formation of heterodimers between the murine and human polypeptides. Although the difference in apparent molecular weight between the human transferrin receptor on the J4 cells and that of CCRF-CEM cells is probably due to a difference in processing of the receptor in L cells (R. Newman *et al.*, unpublished results), it appears that the structural difference cannot be entirely accounted for by glycosylation. The human transferrin receptor of a second independent L cell transformant has a similar molecular weight to that of J4 cells suggesting that the structural alteration of the receptor was not the result of a random event that occurred during the transformation of J4 cells.

Because monoclonal antibodies were available

that specifically blocked the uptake of transferrin-bound Fe mediated by the human and mouse transferrin receptors, respectively, it was possible to investigate whether the transfected human transferrin receptor was functional in the J4 transformants. It was found that both the monoclonal antibody, 42/6, that blocks transferrin binding to the receptor on human cells and monoclonal antibody R17 208 that interferes with Fe uptake by the murine transferrin receptor partially inhibit iron uptake in J4 cells. In combination, however, inhibition of iron uptake by J4 cells was almost complete. It appears, therefore, that the J4 transformants are transporting iron via both the endogenous mouse transferrin receptor and the transfected human transferrin receptor [52]

The demonstration that stable transformants expressing the human transferrin receptor can be obtained by cotransformation of L(tk$^-$) cells with Herpes tk gene and high molecular weight human DNA, together with the recent report by Kavathas and Herzenberg [53] of the feasibility of the transformation of human T cell differentiation antigens, Leu-1 and Leu-2, into L cells suggests that this methodology offers a general viable approach for the isolation of genes encoding cell surface molecules that can be detected on the basis of their antigenicity.

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