Alterations in the Phenotype of Hairy Cells During Culture in the Presence of PHA: Requirement for T Cells

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Culture studies of peripheral blood mononuclear cells from 7 entirely typical cases of hairy cell leukemia showed that after culture in the presence of PHA for 2–5 days, the predominant cell type changed from E S Ig Clg γFcR μFcR hairy cells to an E' S Ig Clg γFcR μFcR population of transformed cells derived from hairy cells. Depletion and readdition experiments demonstrated that cell-to-cell contact with T cells was necessary for the phenotypic change, while several observations indicated that the E' population was not derived from T cells present before culture. The E positivity of the cultured cells was shown to be due to the possession of E receptor not acquired from the culture fluid, but the cells differed from true T cells in lacking both mature and immature T-cell antigens. The relevance of these in vitro observations to the continuing controversy concerning the nature of the hairy cell and to the in vivo fluctuations in immunologic phenotype not infrequently observed in hairy cell leukemia is briefly discussed.

EXTENSIVE surface marker studies in hairy cell leukemia (HCL) have shown that in most cases the pathognomonic hairy cells (HC) synthesize surface (S Ig) and intracytoplasmic (Clg) immunoglobulin of restricted light-chain type, but lack receptors for sheep erythrocytes (E) (reviewed in Cawley et al.). Hairy cells therefore generally display the S Ig E phenotype of B cells, and this had led most authors to conclude that HCL represents a B-cell proliferation (reviewed by Catovsky). However, this simple conclusion has been complicated by the fact that HC possess a number of distinctive features (e.g., content of tartrate-resistant acid phosphatase) and display other characteristics more typical of monocytes. Furthermore, cases of HCL in which the HC display T-cell characteristics may be observed; in other patients, HC may largely disappear to be replaced by E-rosetting cells that differ from normal T cells in a number of respects.

Recently, the simple conclusion that HCL is a B-cell proliferation disorder has been further questioned by the observation of Guglielmi et al. that, after culture in the presence of phytohemagglutinin (PHA), HC may change their surface marker phenotype, acquiring receptors for sheep erythrocytes and reactivity with a rabbit anti-T-cell serum.

In the present article, we confirm the observations of Guglielmi et al. and extend them by showing that the PHA-induced changes require the presence of T cells and that, while the E' HC-derived cells express true E receptors, they do not stain with monoclonal anti-T-cell sera.

MATERIALS AND METHODS

Patients

Seven patients with HCL were involved in the studies. All had entirely typical HCL, and HC formed a high proportion of circulating mononuclear cells in all cases.
Some E' T-cell fractions from normal peripheral blood were obtained in the manner described above, but others were isolated by passing dextran-sedimented blood through cotton wool columns; such fractions contained >95% E' cells.

Culture Studies

Mononuclear cells were cultured at a concentration of 10^6/ml in RPMI 1640 supplemented with 10% fetal calf serum. In initial experiments, cells were cultured in the presence of a range of concentrations of reagent-grade phytohemagglutinin (PHA-M, Gibco, Grand Island, N.Y.) (0.01%, 0.1%, and 0.6%) and purified PHA (PHA-P, Wellcome) (0.1, 1, 2, 3, 4 μg/ml). Less clumping was observed in the PHA-P as compared with the PHA-M cultures, and least clumping was seen at lower concentrations of PHA-P. A concentration of 3 μg/ml of PHA-P was therefore routinely used, but at the same time produced less cell clumping than 4 μg/ml.

In experiments designed to determine the role of autologous or normal allogeneic T cells, separated E' cells were added either directly to purified HC or in ways designed to prevent cell-to-cell contact, e.g., behind dialysis or Millipore (type MF; pore size 0.4μm) membrane or in agar. In the direct readdition experiments, E' cells were added to result in a range of final concentrations (5%-40% E' cells), but the most usual concentration was 20%. In the dialysis membrane experiments, E' cells were enclosed within the membrane and suspended in the pure HC cultures at a concentration resulting in a 1:1 ratio of E':HC. In the Millipore membrane experiments, E' cells or HC were enclosed within the Millipore chamber, which was then immersed in HC or E' cultures, respectively; cell numbers were adjusted to result in an E':HC ratio of 1:1 or 3:1. In the agar experiments, E' cells were incorporated in an agar (0.7%) underlayer and pure HC cultured in liquid medium above the agar; cell numbers were adjusted to produce a range of E':HC ratios of 2:1, 1:1, 1:2. In all these experiments a corresponding culture with directly added E' cells (E':HC, 1:4) showed the phenotypic and morphological changes described later.

In 3 experiments, the supernatants of 3-day PHA-stimulated E' or E' + HC cultures were added for 1-12 hr to fresh HC. In 1 experiment, supernatants from separate 1-5-day cultures of PHA-stimulated E' and E' + HC cultures were added daily to either a single pure HC or HC + E' culture incubated concurrently for 5 days. For these experiments, 10-ml cultures of E' cells or E' (20%) + HC (80%) incubated at 10^6/ml in the presence of PHA-P (3 μg/ml) were employed. The supernatants from gently centrifuged cultures were then added to fresh HC's (10^6 cells) or to centrifuged pellets of pure HC or E' + HC populations cultured in an identical fashion without PHA. Although no changes were observed in the pure HC culture, the HC + E' culture underwent the phenotypic and morphological changes described later, thereby indicating that sufficient PHA was being transferred to enable the described changes to take place, provided E' cells were present.

Monoclonal Antibody Staining

A range of mouse monoclonal antibodies (OKT3, UCHT1, OKT11, Leu-2a, Leu-3a, NA1 34, DA2, OKM1, and HLe-1) were used in an indirect technique employing FITC-labeled sheep anti-mouse Ig or rhodamine-labeled goat anti-mouse Ig. Reactivity was analyzed mainly by fluorescence microscopy, but also by a fluorescence-activated cell sorter (Becton Dickinson FACSII). Negative controls included staining with second layer antisera only and with an irrelevant anti-astrocytoma antibody. The specificity of various antibodies has been described in detail elsewhere but, in brief, OKT3 and UCHT1 specifically stain mature T cells, while OKT11 reacts with the E receptor. Leu-2a and Leu-3a, respectively, stain suppressor and helper subpopulations of mature T cells, and NA1 34 stains most cortical thymocytes, but not mature T cells. DA2 specifically reacts with a nonpolymorphic determinant of HLA-DR, while OKM1 stains human monocytes and certain E' non-T-cells from human blood. HLe-1 specifically stains human hemic cells.

RESULTS

Phenotypic and Morphological Changes in Unfractionated PBM Cultured in the Presence of Mitogen

In the four patients tested, conversion from predominant E' to E populations occurred between 1 and 5 days (Fig. 1). In three of the four patients, S Ig expression was reduced to low levels by 2-5 days (Fig. 1). In the third patient, S Ig continued to be expressed by a high percentage of the cells up to day 6. In repeat experiments in this patient, S Ig' cells were variably reduced after 4-day culture. In the two patients tested, γFcR and μFcR cells were reduced to low levels by days 3-5 (Fig. 1), and similarly, whereas 90% of the cells contained C Ig early in

![Fig. 1](https://www.bloodjournal.org)
culture, by 3.5 and 4.5 days, no Clg was detectable within the cells. In all these cultures, viability remained high (>90%), but formal counts of total cell numbers were not recorded because, particularly in the early stages of culture, clumping made cell enumeration inaccurate.

Microscopic examination of the cultures (Fig. 1) revealed that many morphological HC formed E rosettes early in culture, but later, as few Slg’ cells were seen, morphological HC were replaced by large cells of transformed appearance; 70% of the cells possessed strong TRAP activity. Cultured normal and HCL T cells (E’ cells before culture) contained little or no TRAP activity. PBM cultured in the absence of PHA showed no phenotypic or morphological changes, the predominant population remaining TRAP-positive HC with the E Slg’ γFcR’ phenotype. The phenotypic and morphological changes described above were observed at concentrations of 1% or higher of PHA-P of >1 μg/ml and in the presence of 0.1% and 0.6% PHA-M (but not 0.01%).

PBM cultured in the presence of pokeweed mitogen (1%, Gibco) for up to 5 days showed no morphological or phenotypic changes, and numerous HC persisted throughout the period of culture.

Effect of Removal and Readdition of E-Rosetting Cells Before Culture in the Presence of PHA

In 8 experiments in 5 patients, E’-depleted PBM cultured in the presence of PHA for 5–8 days showed no phenotypic or morphological changes; the cells remained E Slg’ γFcR’ μFcR’, continued to resemble HC, and contained strong TRAP activity.

In 2 different patients, autologous E’ cells were readded to E’-depleted fractions to result in concentrations of between 10% and 30% E’ cells. After culture in the presence of PHA, phenotypic and morphological changes identical with those observed in unfractionated PBM occurred after 4-day culture. Similar results were obtained after readdition of irradiated (2000 rads) autologous E’ cells. In 6 similar experiments involving addition of allogeneic T cells from 4 different normal individuals to E’-depleted HC fractions from 2 different patients, identical phenotypic and morphological changes were again observed. No such changes were observed in the nonstimulated control culture containing readded autologous or normal allogeneic T cells.

Thus, in all these readdition experiments, the predominant population changed from E’ Slg’ Clg’ γFcR’ μFcR’ HC to E’ Slg’ Clg’ γFcR’ μFcR’ cells. Although these were frequently of transformed appearance, they were clearly derived from HC, since they contained moderate TRAP activity (before culture, the HC possessed strong enzyme activity, whereas both transformed and nontransformed E’ fractions possessed no or very faint TRAP).

In contrast, in readdition experiments where cell-to-cell contact was prevented by incorporating the E-rosetting cells in agar (2 experiments in 1 patient), behind dialysis membrane (2 experiments in 2 patients), or Millipore or Nucleopore membranes with large pore diameters (in each case 1 experiment in 2 different patients), no phenotypic or morphological changes were observed, the predominant population remaining E Slg’ Clg’ γFcR’ μFcR’ and strongly TRAP’ HC. In contrast, when fragments of Millipore membrane, together with membrane holder, were incorporated in E’ + HC PHA cultures, the usual phenotypic changes duly occurred. This observation, taken together with the fact that different types of material with low affinity for glycoprotein were used to separate the E’ cells and that serum was included in the culture medium, makes sticking of putatively shed E receptor an unlikely explanation of the failure to detect the phenotypic changes when E’ cells are physically separated from HC.

Similarly, no morphological or phenotypic changes were observed when supernatants from PHA-stimulated E’ and E’ + HC cultures were added to E’-depleted HC fractions.

Analysis of the PHA-Induced Phenotypic Changes by Monoclonal Antibodies

Before culture, HC did not stain with OKT11 or with any of the anti-T-cell antisera tested, but were strongly positive with an anti-human leukocyte antibody and moderately reactive with an anti-HLA-DR antibody (Table 1). After culture (4 experiments in 4 patients) with either autologous or normal allogeneic E’ cells (shown to be T cells by UCHT1 staining), the majority of E-rosetting cells were unstained with the various anti-T-cell sera, but possessed true E receptors since they were strongly reactive with OKT11. The E’ cultured cells were strongly reactive with the anti-HLA-DR antibody and did not stain with the antimonocyte serum (OKM1). Both before and after culture, the mononuclear cells failed to stain with an irrelevant first layer monoclonal antibody or with fluorochrome conjugated second layer, but the strong staining with an anti-human leukocyte antibody constituted a positive control. The persistence of strong staining with HLe1 and HLA-DR make it unlikely that loss of Slg
and γFc receptors can simply be attributed to steric hindrance by putatively shed and passively acquired E receptor. Normal and autologous E+ cells cultured in the presence of PHA remained UCHT1+ OKT11+. Purified (by E depletion) HC cultured both in the presence and absence of PHA remained UCHT1 OKT11.

**DISCUSSION**

In the present study, it is clearly shown that on short-term culture in the presence of PHA, the predominant population in PBM from HCL patients changed from E Slg+ CIg+ γFcR- μFcR+ TRAP+ HC to E Slg- CIg- γFcR- μFcR- TRAP- transformed cells of HC origin. This change was preceded by a phase in which many morphologically typical HC with strong TRAP activity formed E rosettes and during which the mononuclear cells displayed an immunologic phenotype intermediate between the two given above.

Several observations indicated that the appearance of a predominant E+ population did not simply represent an outgrowth of the original T cells present. The conversion to an E+ predominant population occurred in cultures incorporating irradiated T cells; the possibility of selective loss of non-T-cells in the cultures was excluded by the consistently high viability observed. Furthermore, the E+ cells continued to contain significant TRAP activity even when they ceased to resemble HC, and a phase was consistently observed during which typical Slg+ CIg- HC formed E rosettes. Perhaps most significantly, the majority of E+ cells failed to stain with specific monoclonal anti-T-cell sera; in contrast, control PHA-stimulated T-cell cultures were positive with these anti-T-cell sera and completely lacked TRAP activity.

The possibility that the E receptor detected on the HC-derived mononuclear cells was passively acquired receptor shed by PHA-stimulated T cells was excluded by careful experiments involving repeated addition of supernatant and by the fact that no E+ conversion occurred when T cells were readded in agar or behind Millipore membrane. Also, the E-rosetting was shown to be detecting true E receptor, since the E+ cells stained with a monoclonal antibody against the E receptor.

The present results confirm and extend those of Guglielmi et al.,7 but our experiments with isolated cell populations showed that the intimate presence of T cells is essential for the phenotypic changes to occur. Furthermore, our studies with monoclonal anti-T-cell sera indicate that, although the HC-derived cells expressed true E receptor and lost B-cell characteristics such as Slg and CIg, they lacked antigens present on normal and PHA-transformed T cells and most thymic cortical cells. The acquisition of E receptor cannot therefore be unequivocally regarded as a T-cell feature of HC, more especially because a population of E+ non-T mononuclear cells is now recognized in normal peripheral blood.19,20 The latter population is probably heterogeneous, and some may be of monocytic lineage since they stain with an antimonocyte antibody (OKM1).19 However, although HC possess certain "monocytic" characteristics, they cannot readily be related to this normal E+ monocytic population since the E+ HC-derived cells failed to stain with OKM1. Perhaps, then, the present data relate HC to the E+, non-T, nonmonocytic population of normal blood.

Whatever the significance of our findings in relation to the nature of the HC, they provide an in vitro counterpart of the emergence of a predominant E+
phase in vivo and raise the possibility that the stress situations that may provoke the in vivo change may act via the normal T cells present.

It is being increasingly recognized that different types of leukemic cell may be converted from nonfunctional to functional states by appropriate in vitro stimuli. These observations, taken together with the highly active appearance of the E+HC-derived cells, suggests that such cells may be functionally active; this possibility is currently under investigation.

REFERENCES

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