Hand-Mirror Lymphocytes in Infectious Mononucleosis

By William J. Thomas, Kazue Yasaka, D. M. Strong, C. M. Woodruff, Sanford A. Stass, and Harold R. Schumacher

The identification of a morphologically unique lymphocyte in the peripheral blood of patients with infectious mononucleosis (IM) led to additional studies. Examination of Wright-stained smears of EDTA-anticoagulated blood obtained from 25 patients with IM at presentation revealed significantly increased (p < 0.0001) percentages of hand-mirror lymphocytes (HML) (mean 9.2%) compared with normal controls (mean 2.7%) or controls with nonspecific viral syndromes (mean 2.2%). Follow-up blood samples obtained on 10 of these patients demonstrated a marked increase in the HML count (mean 23.1%) that coincided with the onset of recovery. E-rosette separation of Ficoll-Hypaque-derived peripheral blood lymphocytes from 5 patients with early recovery IM showed the HML to be present almost exclusively in the T-cell population, representing about 25% of the T cells. Identical procedures on 5 controls showed less than 5% HML in the T-cell sample. Cytocentrifugation supported a T-cell derivation for HML. Electron microscopic examination of HML in IM demonstrated that these cells have unique ultrastructural features that may be related to functions of cellular attachment and cytotoxicity.

Infectious mononucleosis (IM) is caused by Epstein-Barr virus (EBV). The lymphoid hyperplasia and atypical lymphocytosis observed in the disorder represent a host reaction of increased numbers of activated T lymphocytes directed against EBV-infected cells. Numerous morphological DNA-synthetic, and cell culture studies have stressed the similarity between IM and malignant lymphoproliferative disease. It has been suggested that differentiation between these disorders is based primarily on the self-limited reversible nature of IM. Presumably, there are control mechanisms operating in IM that are important in limiting the lymphoproliferation. Knowledge of these mechanisms would provide important insights into the processes of immune surveillance and malignant transformation in man. During the acute phase of their illness, patients with IM harbor killer cells that exhibit specific in vitro cytotoxicity for Epstein-Barr-viral-genome containing B cells. The exact immune mechanisms and cell types responsible for control of EBV-altered B cells in vivo are not known, although they are thought to reside in the expanded T-cell population. We have identified increased numbers of a morphologically unique lymphocyte, the hand-mirror lymphocyte (HML), in the peripheral blood of patients with IM. Since the hand-mirror form has been implicated as an important structure in experimental lymphocyte cytoxicity, we thought it important to examine more closely HML in IM.

Materials and Methods

Twenty-five adolescents and young adults with IM comprised the study population. Diagnosis was based on a typical clinical syndrome, characteristic peripheral blood findings, and a positive serologic test for mononuclosis using the Monosicon DRI-DOT test (Oragen, West Orange, N.J.). Twenty-five healthy adolescents and 25 with clinically diagnosed viral syndromes served as controls. Blood was drawn on all patients on the day they first presented to the clinic for evaluation. Serial blood samples were obtained on 10 patients with infectious mononucleosis during the acute phase of the illness when symptoms were maximal, at the onset of recovery when symptoms began to abate, and when recovery was completed as evidenced by normalization of symptoms and physical findings. Complete blood counts were performed on each sample within an hour after collection. Wright-stained smears, prepared from EDTA anticoagulated blood, were examined by two physicians (C.W. and H.S.) who had no knowledge of the patients or the stage of their illness, and the percentages of lymphocytes and atypical lymphocytes were determined by a 300-cell differential count. Atypical lymphocytes were arbitrarily subclassified into Downey type and hand-mirror type. Hand-mirror lymphocytes were those cells containing a nucleus in the "mirror" portion with an opposed cytoplasmic tail, termed a uropod, forming the "handle" portion (Fig. 1). Peripheral smears from 20 patients with IM were investigated cytchemically for periodic acid Schiff (PAS), Sudan Black B, and myeloperoxidase positivity and for beta-glucuronidase, naphthol-AS-D chloracetate esterase, alpha-naphthyl butyrate esterase, tartrate-resistant acid phosphatase, and tartrate-sensitive acid phosphatase activity.

Electron microscopy was performed onuffy coat preparations from 20 patients. The buffy coat button was placed in 2.5% glutaraldehyde and processed as previously described. Lymphocyte suspensions were prepared from peripheral blood samples of five patients with early recovery IM by techniques described in detail elsewhere. Briefly, mononuclear cells were obtained by Ficoll-Hypaque density-gradient separation of defibrinated whole blood. The mononuclear peripheral blood leukocytes (MPBL), thus obtained, were mixed with sheep erythrocytes (SRBC) and allowed to form rosettes at 4°C. Ten milliliters of SRBC at a concentration of 4 x 10^8/ml were mixed with 10 ml fetal calf serum (FCS) and 5 ml of MPBL at 4 x 10^8/ml. After 30 min incubation at 4°C, the mixture was layered onto Ficoll-Hypaque and centrifuged at 800 g for 20 min. Theuffy coat, containing 5 x 10^7 rosette-forming cells/mL, was taken as the initial rosette fraction. The Buffy coat was washed three times with cold phosphate-buffered saline (PBS) to remove nonadherent SRBC, centrifuged, and resuspended in cold PBS for subsequent experiments. The number of peripheral blood lymphocytes that formed rosettes (E-rosettes) with sheep erythrocytes at 4°C was determined for each sample.

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Fig. 1. Hand-mirror lymphocytes from a patient with IM during the early recovery phase of the illness. Thirty-three percent of this patient's lymphocytes possessed the characteristic hand-mirror configuration. (Wright-stained smear, original magnification x320).

centrifuged for 30 min at 550 g. The pellet was rich in rosette-forming cells (E+ cells) and contained less than 2% nonrosetting cells (E- cells). The interface was a mixed population of cells, composed of a few percent contaminating E+ cells, B cells as determined by surface membrane immunoglobulin (Smlg), monocytes (as determined by Wright and esterase stains), and null cells (those E- cells after monocyte depletion having no Smlg or T markers). Wright-stained slides were prepared from each of the different samples (MPBL, interface, E+ pellet), and the percentage of HML was determined by a 300-cell count.

The statistical method utilized to determine significant differences in our results was the two-tailed Student's t test.

RESULTS

The blood from 25 patients with newly diagnosed IM showed significantly increased ($p < 0.0001$) numbers of HML (mean 9.2%, range 1.2%-22.5%) when compared with normal controls (mean 2.7%, range 0%-5.8%) or viral controls (mean 2.2%, range 0%-5.7%). Viral and normal controls did not differ significantly. Serial blood samples were obtained on 10 patients with infectious mononucleosis during convalescence. The total lymphocyte count and atypical lymphocyte count did not change significantly between the acute symptomatic and early convalescent phases of the illness. However, the number of HML varied during the course of the disease. The mean percentage of HML was 6.7% (range 2.7%-13.9%) when these 10 patients first presented to the clinic for evaluation. It remained at this level during the initial phase of their illness when symptoms were maximal. A significant ($p < 0.0001$) rise in the percentage of HML (mean 23.1%, range 14.5%-33.1%) was noted to occur at the onset of recovery when the patients reported the initial improvement in symptoms. There was no other laboratory or physical clue to indicate that the patient was getting better. Following the peak HML count, the signs and symptoms of mononucleosis steadily remitted, and the percentage of hand mirror lymphocytes returned to control levels. Figures 2 and 3 summarize the findings of these 10 patients. Statistical analysis of these data show that the significant increase ($p < 0.0001$) in HML that occurs between the acute and early convalescent phases of illness coincides with a significant decrease ($p < 0.0025$) in Downey-type atypical lymphocytes.

In order to characterize the HML more precisely, cytochemistries were performed. HML exhibited moderate to marked tartrate-sensitive acid phosphatase activity. Beta-glucuronidase was generally less intense, but some HML showed a strong focal pattern in the uropod. PAS staining was variable; most cells were negative, but some HML had block positivity in the uropod. Staining of HML for Sudan Black B, myeloperoxidase, naphthol-AS-D chloracetate esterase, alpha-naphthyl butyrate esterase, and tartrate-resistant acid phosphatase was negative.

E-rosette separation of Ficoll-Hypaque-derived peripheral blood lymphocytes from 5 patients with early recovery IM showed the HML to be present almost exclusively in the T-cell population, representing 25% of T cells (range 21%-30%). The interface mixture of Smlg+ B lymphocytes, null cells, and monocytes contained 1%-4% HML (Table 1). Analy-
The pinocytosis and micropinocytosis seemed to be more pronounced in the uropod. Figures 4A and B, transmission electron micrographs of a hand-mirror lymphocyte from a patient with infectious mononucleosis, demonstrate many of the previously described features of this cell.

**DISCUSSION**

The finding of increased numbers of hand-mirror lymphocytes (HML) in patients with infectious mononucleosis (IM) and their correlation with the phase of disease suggested that these cells have a role in the self-limitation of IM. The morphology of the HML, which consists of an eccentric nucleus and a cytoplasmic tail, termed a uropod, is distinctly different from the classical description of atypical lymphocytes of Downey. The first description of the hand-mirror cell was Lewis’s observation of moving rat lymphocytes in tissue culture. Hand-mirror cells were associated with cells of human lymphocytic origin when they were described by Rich, Wintrobe, and Lewis in tissue culture of normal lymph nodes, infectious mononucleosis, chronic lymphatic leukemia, and acute lymphoblastic leukemia. McFarland et al. demonstrated the immunologic importance of the uropod. They showed, by microcinematography, intimate attachment of the uropod with macrophages, lymphocytes, and cellular debris during the mixed lymphocyte reaction. Additional evidence that uropod formation is associated with immunologic activation of lymphocytes is supplied by several other studies. Biberfeld found that cultures of PHA-stimulated human peripheral blood lymphocytes develop increasing numbers of hand-mirror forms, up to 20%, compared to the 5% level found in unstimulated cultures. In studies of guinea pig lymphocytes, Rosenthal and Rosenstreich found uropod-bearing cells to be most numerous among the highly antigen-reactive peritoneal exudate lymphocytes and significantly less numerous among the less reactive lymph node lymphocytes. Thymus cells, which contained immature nonactivated cells, had the fewest HML. In their study, passage of lymphocytes through adherence columns that preferentially removed B cells resulted in an increased proportion of uropod-positive cells. In addition, there was no detectable surface immunoglobulins on the cells that formed uropods. Thus, these cells were assigned to the thymus-derived cell line.

Our investigation of HML in IM confirm the impression of previous authors that HML represent a subset of immunologically activated thymus-derived lymphocytes. HML were observed infrequently in normal controls and in patients with unidentified viral syndromes. Their presence in significantly increased

**Table 1. Distribution of Hand-Mirror Lymphocytes After Ficoll-Hypaque, E-Rosette Separation of Peripheral Blood From Five Patients With Early Recovery IM and From Five Normal Controls**

<table>
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<th>Percent Hand-Mirror Lymphocytes</th>
<th>Peripheral Smear</th>
<th>MPBL*</th>
<th>Interface †</th>
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*MPBL, mononuclear peripheral blood leukocytes.
†Interface: SmIg⁺ B lymphocytes, monocytes, null cells, rare contaminating E⁻ cells.
‡E′ pellet: SmIg E-rosette-forming T lymphocytes, <2% nonrosetting E cells.
numbers in IM, an illness that represents an intense immunologic reaction against EBV infection, suggests an immunologic role for these cells. E-rosette separation of Ficoll-Hypaque-derived peripheral blood lymphocytes showed the HML to be present almost exclusively in the SmIg T-cell population. The cytochemical pattern of acid phosphatase and β-glucuronidase positivity is supportive of a T-cell derivation of HML. The cytologic and ultrastructural features of HML in IM were found to be identical to those observed among HML in lymphocyte-lymphocyte, lymphocyte-macrophage, and lymphocyte-target cell interactions.

The accuracy of our results comparing the numbers of HML in IM with control groups may be questioned because of a potential prejudicial element present in the design of the study. Although the peripheral blood smears were labeled in code to prevent the examiners from knowing the diagnosis, it was not possible to completely blind the study, since a slide with a large population of atypical lymphocytes immediately suggests a diagnosis of IM. However, data from two parts of the study that were not affected by this potential observer bias indicate that our slide examiners were successful in accurately counting HML. Five patients with early recovery IM and five normal controls were correctly identified by determining the numbers of HML present in lymphocyte suspensions. Observer bias was not a problem in this part of the study because slides of the lymphocyte suspensions did not exhibit the cellular detail that would permit the identification of increased numbers of atypical lymphocytes. In another part of the study, the peripheral blood smears of patients with early recovery IM were differentiated from the acute illness slides by the increased numbers of HML present. The identification of increased numbers of atypical lymphocytes did not bias these results because there were equal numbers present in both samples.

We investigated the role of HML in IM by analysis of lymphocyte populations in patients during the course of their illness. The total lymphocyte count and atypical lymphocyte count did not change between the acute symptomatic and early convalescent periods of the disease. However, the number of HML increased sharply during the early phase of convalescence. If total atypical lymphocytes are separated into the classic Downey type and the unique hand-mirror type, it

Fig. 4. Transmission electron micrograph of a hand-mirror lymphocyte from a patient with IM. (A) Note typical cellular configuration with a mature clefted nucleus (N) in the "mirror" portion of the cell and the cytoplasmic uropod (u) forming the "handle" portion (original magnification x 7800). (B) High-power magnification of the uropod showing elongated mitochondria (m), microtubules (mt) extending from the satellite bodies of the centriole (c) toward the nucleus, and the prominent terminal microspikes (ms). Numerous pinocytotic vesicles and vacuoles are present in this region of the cell (original magnification x 23,750).
appears that the increase in HML occurs at the expense of a decrease in Downey atypical lymphocytes. Since this occurs at a time coincident with the onset of recovery, it is tempting to propose that the HML represent a structural modification of the T-lymphocyte population that has functional importance in the control of EBV-induced lymphoproliferation. In vitro lymphocyte cytotoxicity experiments provide insights into the possible functions and mechanisms of HML. Cytotoxicity of PHA-stimulated human peripheral blood lymphocytes for Chang liver cells has been shown to involve direct contact between lymphocyte and target cell, often in the region of the uropod. Unstimulated peripheral blood lymphocytes are likewise cytotoxic for antibody-coated target cells by a mechanism requiring intimate contact between killer and target cells. Uropod formation is the prominent morphological feature of the lymphocytes adherent to the target cells in these experiments.

A recent study on blood lymphocytes in IM has demonstrated the presence of a subpopulation of activated T lymphocytes that has acquired the capacity for natural attachment (NA), i.e., attachment in vitro to various human normal and malignant cells. In this study, it was not determined whether NA was a nonspecific result of T-cell activation or whether it was important in killer–target cell interactions.

The in vitro lymphocyte cytotoxicity data and our morphological observations suggest that HML in IM may play a role in cellular attachment and cytotoxicity. However, alternative hypotheses are possible. The hand-mirror form has been associated with lymphocyte motility. It may represent the passive accumulation of cytoplasmic structures in the trailing part of a motile lymphocyte or it may be secondary to interactions between membrane proteins and cytoplasmic contractile proteins that precede cell movement.

Lymphocytes have also been observed to form uropods after exposure to antigen–antibody complexes. Furthermore, when antigenic molecules such as ferritin are added to lymphocyte suspensions, they are rapidly taken up and concentrated in the uropod region. Thus, the uropod has been considered to be an area specialized for endocytosis.

The relationship between these observed phenomena of hand-mirror formation and the presence of increased numbers of HML in IM is not clear. HML in IM may be a nonspecific manifestation of lymphocyte activation, may be directly involved in cytotoxicity, or may perform some secondary role in the body's response to EBV. It will be important to correlate the variation in lymphocyte morphology observed in our study with changes in lymphocyte function that occur in IM. This would lead to a better understanding of the role of HML in IM and the possible elucidation of the mechanisms operative in the control of EBV induced lymphoproliferation.

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