Cell Migration Inhibition in Human Lymphomas
Using Lymph Node and Cell Line Antigens

By Marta Braun, Luisa Sen, Alois E. Bachmann, and A. Pavlovsky

In an attempt to demonstrate in vitro reactions against autologous and allogenic lymph node (LN) extracts in lymphoma patients, 15 cases were studied using as controls two normal LN extracts and leukocytes from 17 blood donors. The techniques applied were migration inhibition tests carried out directly on peripheral leukocytes and indirectly using lymphocyte culture supernatants on guinea pig peritoneal macrophages. The antigens (Ag) used were partially purified saline extracts of neoplastic LN, cultured neoplastic cells of lymphosarcoma (LS) origin, and normal LN. It was observed that autologous LN extracts elicited migration inhibition in the four cases of LS studied and in four out of six cases of Hodgkin's disease (HD), while normal LN extracts gave negative results in these patients. Positive reactions were observed in four out of six LS cases using allogenic LS extracts, either from LN or from cell lines. No cross-reactions could be demonstrated in HD. All Ag gave negative results with leukocytes from normal blood donors. The positive autologous reaction suggests the presence of a tumor Ag in neoplastic LN, while the immunologic cross-reaction between some of the LS patients would point to a common Ag, persisting in at least one of the LS cell lines and perhaps related to a virus.

Although there is constantly growing evidence in favor of a host immunologic reaction against its own tumor, the possibility of demonstrating such a response is obviously more difficult in man than in animals. Moreover, the mechanisms involved in this immunologic reaction are still partially obscure. Although circulating antibodies have been demonstrated in patients bearing malignant neoplasms, a greater importance is being attributed to cellular immunity in the protection against tumors.1-3

It is generally accepted that, in animals, chemically induced tumors may give rise to strong immunity, but the antigenic determinants are specific for each tumor even when induced by the same carcinogen in inbred animals.4 On the contrary, tumors caused by oncogenic viruses lead to cross-immunity,5 and the same virus can produce common tumor-specific antigens (Ag) in cells from two different species.6

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The cell migration inhibition technique has been used as an in vitro test for the detection of delayed hypersensitivity (DH) in man. This method is based on the fact that the migration of white blood cells from sensitive donors out of capillary tubes is inhibited by the presence of the specific Ag in the culture medium; this inhibition is thought to be caused by the release of a migration inhibitory factor (MIF) by sensitive lymphocytes in the presence of the Ag. As human MIF is able to inhibit guinea pig macrophage migration, an indirect technique was developed in which human leukocyte culture supernatants were tested for MIF activity using guinea pig peritoneal cells as migrating populations.

The main advantage of using in vitro tests to study DH in man is based upon the fact that no intradermal administration of Ag (tumor material) is required.

By applying direct and indirect cell migration inhibition tests, evidence was found in patients bearing lymphomas of DH against extracts of their own malignant lymph nodes (LN). It seemed of interest to continue this work, studying DH in different types of lymphomas and investigating the presence of cross-reactions between patients bearing the same kind of tumor. It also seemed interesting to study the cellular migration of these patients in the presence of extracts prepared from two different cell lines originating from a human lymphosarcoma (LS).

MATERIALS AND METHODS

A total of 15 patients bearing LN malignancies were studied, using as controls LN and peripheral blood from two patients undergoing surgery for benign diseases, as well as blood from 17 normal donors. Lymphoma patients included: eight LS cases, four Hodgkin’s disease (HD) cases, one acute lymphatic leukemia (ALL) case, and two metastatic LN (case 4 from epithelial origin and case 15 from a mixed parotid tumor). Normal LN were obtained in the course of surgery for inguinal hernia (case 5) and gall bladder lithiasis (case 6).

Direct Test

Peripheral white blood cell migration inhibition tests were carried out following a slightly modified Bendixen and Söborg technique. Buffy coat cells were harvested after dextran (mol wt 60,000) sedimentation and were packed in capillary tubes; these were placed in pairs in lucite chambers that were closed with glass slides and filled with TC 199 medium, heparin (20 IU/ml), and kanamycin (0.04 mg/ml). Ag was added to all but one or two chambers.

Indirect Test

Indirect tests were carried out in two steps. Preparation of Lymphocyte Culture Supernatants: Total peripheral leukocytes were incubated for 24 hr in TC 199 medium with autologous plasma (40–50%), heparin (25 IU/ml), and kanamycin (0.04 mg/ml); cell concentration was approximately 5 × 10^6 cells/ml. Cultures were set up in 10-ml tubes containing 2 ml of cell suspension each. Ag (50 μg/ml) was added to all but one or two tubes.

After incubation cultures were spun gently (5 min, 100 g), and supernatants were discarded. Cells were washed once in Hank’s solution, resuspended in 2 ml of fresh medium without Ag, and cultured for another 24 hr. Heterologous plasma was never used to supplement TC 199 medium. Cultures were centrifuged (10 min, 1,500 g), and supernatants were immediately assayed for MIF activity.
**Test for MIF Activity:** Four or 5 days before the test, a guinea pig was injected i.p. with 10 ml of sterile liquid mineral oil. The peritoneal cells were harvested and washed three times with heparinized Hank's balanced salt solution and resuspended in 5 volumes of TC 199. Capillary tubes were filled with the cell suspension, heat sealed at one end, and centrifuged (3 min, 150 g). Capillaries were cut below the cell/liquid interphase and placed in lucite chambers. Chambers were filled with the supernatants obtained as specified above and closed with a glass slide sealed with silicone grease.

**Migration Index**

All chambers, in both direct and indirect tests, were incubated for 24 hr at 37°C. The migration areas were measured by using a microscope with a reticulum inserted in the ocular lens (total magnification 12.5 ×). Migration indices (MI) were calculated by applying the following formula:

\[
MI = \frac{\text{Average area of migration with Ag}}{\text{Average area of migration without Ag}} \times 100
\]

Duplicate determinations were carried out in all tests. If the pairs differed by more than 10%, the test was discarded and repeated if possible. A MI of less than 80% was considered positive.

**Antigens**

Saline extracts of biopsied LN were prepared as described elsewhere; LN were homogenized and sonicated (Mullard MSE, 20 kc/s, 10 min) in isotonic buffered saline, pH 7.2, and lipids were removed with one-half volume of chloroform. The aqueous phase was adjusted to 0.1 mg of proteins/ml with TC 199, and the Ag were fractionated and stored at −20 with kanamycin. All steps were carried out aseptically and at 4°C.

The GH7 and LDLT cell lines were established from a patient who died of LS; GH7 was obtained from LN cells, while LDLT line was derived from peripheral lymphocytes of the same patient, transformed by the addition of DNA extracted from GH7 cells.

**Table 1. Migration Index Using Autologous Lymph Node Extracts**

<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnosis</th>
<th>Direct Test</th>
<th>Indirect Test</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Patient</td>
<td>Control</td>
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<tr>
<td>2</td>
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<td>3</td>
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<tr>
<td>Mean</td>
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<tr>
<td>1</td>
<td>Hodkin’s disease</td>
<td>49</td>
<td>111</td>
</tr>
<tr>
<td>13</td>
<td>Hodkin’s disease</td>
<td>72</td>
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<td>97</td>
<td>135</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>76</td>
<td>119</td>
</tr>
<tr>
<td>11</td>
<td>A.L.L.*</td>
<td>202</td>
<td>101</td>
</tr>
<tr>
<td>4</td>
<td>Metastasis†</td>
<td>56</td>
<td>108</td>
</tr>
<tr>
<td>15</td>
<td>Metastasis‡</td>
<td>100</td>
<td>106</td>
</tr>
<tr>
<td>5</td>
<td>Normal</td>
<td>109</td>
<td>128</td>
</tr>
<tr>
<td>6</td>
<td>Normal</td>
<td>108</td>
<td>106</td>
</tr>
</tbody>
</table>

*A.L.L., Acute lymphatic leukemia.
†Metastasis from a mixed malignant parotid tumor.
‡Metastasis of epithelial origin.
The cells from both lines are morphologically, cytochemically, and chromosomally identical, but viruslike particles were visualized only in the LDLT line cells. Extracts from GH7 and LDLT cells were obtained by sonicating packed cells in 1 volume of buffered saline; lipid extraction, dilution, and storing was done as with the LN preparations.

Each Ag was used at a concentration of 50 μg/ml of culture medium and was tested simultaneously upon leukocytes from both a patient and a normal donor.

RESULTS

Cellular Migration Using Autologous Extracts

The results of 13 tests using autologous Ag are summarized in Table 1. As can be seen in all four cases of LS tested, inhibition of cell migration was observed in the presence of autologous extracts; with the direct test MI averaged 52, the values ranging from 27 to 80; with the indirect test MI averaged 34, the values ranging from 59 to 80. In normal controls, these same extracts often produced increased migration of white blood cells in the direct test but had no effect by the indirect test, the mean MI being 122 and 101 for the two tests, respectively.

Two out of four extracts of HD gave positive reaction in autologous tests; three extracts produced enhanced migration on normal controls. The average MI for patients and controls was 76 and 119, respectively. One of the metastatic LN extracts also gave positive reaction on autologous leukocytes.

Both normal LN gave MI above 100 when tested on patient’s and control leukocytes (direct test) and were negative in indirect tests. In the case of ALL, there was a great increase of the white blood cell migration with the autologous LN extract.

Cellular Migration Using Allogeneic Extracts

A total of 11 Ag were assayed on the leukocytes of six patients with LS, four with HD, one with ALL, and one with a metastatic malignant mixed tumor.

Lymphosarcoma Cases

Of the six cases studied, four gave positive results at least with one of the allogeneic Ag (Cases 2, 7, 8, and 10). Of these four cases, three were also positive when challenged with autologous Ag (Cases 2, 7, and 10, Table 1), while the remaining case was not tested. Unfortunately, the negative cases could not be tested with autologous extracts because no LN biopsy could be performed. As can be seen in Table 2, Ag 3, 19, and LDLT gave positive results in the majority of the patients and normal or increased MI when tested on normal leukocytes. Ag 7 and GH7 were negative in all cases.

Extracts from normal (Ag 5 and 6) and metastatic (Ag 4) LN gave consistently negative results. No extracts from LN with HD were used on these patients.

Hodgkin’s Disease Patients

Using three different Ag from allogenic LN with HD, no clear-cut positive reaction was elicited when tried on three patients with HD, although their MI
were always lower than the respective controls. With all other Ag (LS nodes and cell lines), the results were consistently negative. Furthermore, normal LN extracts gave rise to slightly increased leukocyte migration, as in their controls.

Other Diseases

One ALL and one mixed parotid tumor were challenged with AG from LS and normal LN, and GH7 and LDLT cell lines, giving negative results in all cases.

DISCUSSION

Although the validity of the migration inhibition test, when carried out with buffy coat human leukocytes, has been questioned by some authors, others

<table>
<thead>
<tr>
<th>Case</th>
<th>Extract</th>
<th>Direct Test</th>
<th>Indirect Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Patient</td>
<td>Control</td>
</tr>
<tr>
<td>2</td>
<td>Ag 3 (LS)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>Ag 19 (LS)</td>
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<td>—</td>
</tr>
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<td>Ag LDLT</td>
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<td>—</td>
</tr>
<tr>
<td>7</td>
<td>Ag 3 (LS)</td>
<td>74</td>
<td>118</td>
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<td>7</td>
<td>Ag 19 (LS)</td>
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</tr>
<tr>
<td>7</td>
<td>Ag LDLT</td>
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<td>113</td>
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<tr>
<td>7</td>
<td>Ag 5 (Normal)</td>
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<td>116</td>
</tr>
<tr>
<td>8</td>
<td>Ag 3 (LS)</td>
<td>74</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>Ag 19 (LS)</td>
<td>83</td>
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</tr>
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<tr>
<td>8</td>
<td>Ag 5 (Normal)</td>
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<td>9</td>
<td>Ag 3 (LS)</td>
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<td>Ag 7 (LS)</td>
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<td>Ag LDLT</td>
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<td>9</td>
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<td>10</td>
<td>Ag GH7</td>
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<tr>
<td>10</td>
<td>Ag 6 (Normal)</td>
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<td>Ag LDLT</td>
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<td>14</td>
<td>Ag GH7</td>
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<tr>
<td>14</td>
<td>Ag 6 (Normal)</td>
<td>115</td>
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</tr>
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</table>

*LS Lymphosarcoma.
†Ag 3 gave consistently negative results on controls (six direct and three indirect tests not listed in this Table).
have insisted on its reliability.\textsuperscript{14,15} Many modifications of the peritoneal cell migration test have been proposed, i.e., Thor et al. used dialyzed and concentrated supernatants of purified peripheral lymphocytes on guinea pig macrophages.\textsuperscript{9} Although Thor’s method ascertains the ability of human MIF to act upon guinea pig cells, it is too complicated to use in clinical trials. Our method is based on the initial reports that described MIF,\textsuperscript{8} but Lamelin et al. also proved MIF activity in unconcentrated supernatants of pulse-stimulated lymphocyte cultures.\textsuperscript{16} The method we used has the following advantages: it can be carried out with small amounts of blood (10 ml are enough to test two or more Ag); it may be used simultaneously with the direct (Söborg) test, so that the results can be checked in each instance; the cells migrate in the absence of Ag, so that any direct cytotoxicity of the Ag can be ruled out; and it is reasonably simple.

The migration inhibition observed in our patients in the presence of their own malignant LN extracts is a specific reaction;\textsuperscript{10} moreover, it is not caused by an organ-specific Ag. The migration of white blood cells from patients, as well as from normal blood donors, was slightly increased in the presence of normal LN extracts. All these findings suggest the presence of an antigen(s) in malignant LN; this Ag is able to elicit DH reactions in the lymphoma patients, whereas normal blood donors consistently show negative results.

The presence of DH reactions in cancer patients has also been demonstrated in vivo by Hughes and Lytton\textsuperscript{17} and by Stewart\textsuperscript{18} using saline extracts of autologous tumors and delayed skin reactions in different types of malignant tumors. This reaction was present in 26\% of their patients and was not due to bacterial contaminations of the Ag.\textsuperscript{18} Delayed skin reactions were also demonstrated in lymphomas; Herbermann and Oren\textsuperscript{19} found positive results in patients with many types of lymphomas, and Fass et al.,\textsuperscript{20} in Burkitt lymphoma, found similar reactions against autologous extracts. Using an in vitro technique for the detection of DH, Hellström et al. were able to demonstrate lymphocytotoxicity by a colony inhibition test in patients with many kinds of malignant tumors;\textsuperscript{21} Bubeník et al., using a similar technique, demonstrated DH in urinary bladder carcinoma.\textsuperscript{22} In our laboratory, it has been possible to demonstrate lymphocyte transformation in cultures, in the presence of neoplastic LN autologous extracts, in different types of lymphomas.\textsuperscript{23}

As for the migration inhibition test, it has been used successfully for the detection of tumor immunity in animals.\textsuperscript{15,24–27} In man, Andersen et al. have obtained results similar to ours with autologous extracts of mammary tumors.\textsuperscript{28} Recently, Wolberg and Goelzer\textsuperscript{29} have also demonstrated inhibition of migration in different types of neoplasia; although their interpretation differs from ours, their results can also be explained by postulating that the factor responsible for this phenomenon is a tumoral Ag.

In our results, it is interesting to note that out of four patients giving negative results with their autologous Ag, three have had the worst clinical evolution, two of them dying (1 mo and 5 mo after the test) and the third having now a generalized form of the disease. All our patients were not studied in the last stage of the disease but were usually tested when first seen and virgin of
treatment and are moderately well now. These observations are in accordance
with those of Fass et al.\(^2\) in Burkitt lymphoma patients, in whom longer re-
missions were associated with positive skin reactions, and with those of
Herbermann and Oren\(^1\) who found that negative skin reactions in patients
with ALL tended to become positive when remissions occurred after treatment.

The presence of cross-reactions between some of the LS cases suggests the
presence of a common antigenic factor(s) that would be located in neoplastic
LN and would persist in at least one of the LS cell lines. Common Ag have
been found in human cancer cells by other workers. Hellström et al.\(^3\) found
cross-reactions in patients with colon carcinoma, neuroblastoma, carcinoma
of the breast and lung, sarcomas, and other types of malignant tumors. Morton
and Eilber\(^4\) found that sera of sarcoma patients cross-reacted with cells de-
rived from different types of sarcomas, and as similar reactions were also
found in some relatives of the patients,\(^5\) they postulated the presence of a
virus. Grifoni et al.\(^6\) found passive hemagglutination antibodies in the sera
of HD patients; these antibodies reacted with the autologous and some of
the HD allogenic LN extracts, and they also found antibodies against normal LN.
In the present work, clear-cut cross-reactions in HD patients were not found;
this, however, must be repeated with a larger number of cases.

It can be noted that not all of the patients that reacted with allogenic ex-
tracts followed a common pattern in their responses, e.g., patient 2 gave a
very positive reaction with Ag 3, 19, and LDLT, while patient 8 gave a posi-
tive reaction with Ag LDLT (MI, 49) and a borderline reaction with Ag 3 and
19 (MI, 74 and 83, respectively). This difference could be due to several causes:
although protein content in all extracts was the same, the Ag concentration was
unknown and may have varied considerably; there may have been differences
in the individual immunologic responses, due to variations in the disease and/
or treatment in some cases; and more than one antigenic determinant could
have been present in each extract, the patients reacting independently against
each one of them.

The slight increase of the patients MI in the presence of normal LN extracts
is comparable to that shown by control leukocytes in the presence of normal
and neoplastic extracts. Steiner and Watne\(^7\) also found increased migration,
both in rat and mouse, in the presence of normal tissue extracts; this effect,
however, was not reported by other workers.\(^8\) Further observations are
necessary to elucidate whether this effect is caused by the enrichment of the
culture medium by the addition of homologous proteins or by a factor(s)
present in LN.

On the basis of the experimental studies showing that common Ag are
present in virus-induced tumors but not in chemically-induced tumors, and
considering that viruslike particles have been seen in LDLT cells, it may be
postulated that a possible common virus may be present in some of our LS
patients either as a passenger virus or as an oncogenic agent. To confirm this
working hypothesis, further experiments applying migration inhibition and
other techniques will be carried out using cell extracts, including Ag obtained
from Burkitt lymphoma cells containing EB virus particles.
CELL MIGRATION INHIBITION

REFERENCES


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