In Vitro Transfer of Cellular Immunity against Nasopharyngeal Carcinoma Using Transfer Factor from Donors with Epstein-Barr Virus Antibody Activity

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SUMMARY

The peripheral leukocyte migration inhibition assay has been adapted as an in vitro assay of cell-mediated immunity to a soluble antigen extract of nasopharyngeal carcinoma (NPC) and as an assay for transfer factor (TF) activity in this system. Ten normal individuals with positive Epstein-Barr virus (EBV) antibody titers (1:16 or greater) demonstrated leukocyte migration inhibition (mean ± S.E.) of 36.8 ± 3.4%, at an NPC antigen concentration of 50 μg/ml, compared to a mean migration inhibition of 13.8 ± 5.3% in 10 EBV-negative normal subjects (EBV antibody titer, < 1:4), and this difference was statistically significant (p < 0.002). Four NPC patients in remission demonstrated a mean migration inhibition of 28.8 ± 4.0% when their leukocytes were incubated in the presence of NPC antigen while 4 patients in relapse did not demonstrate migration inhibition (mean 4.3 ± 5.0%), and this difference was significant (p < 0.03). TF derived from EBV-positive donor leukocytes converted leukocytes of EBV-negative normal recipients to reactivity against the NPC antigen in 7 of 9 cases, and this response was highly significant (p < 0.002). The ability of TF to convey reactivity was specific and correlated with EBV humoral immunity in the TF donor. TF also conferred similar reactivity upon the leukocytes of 2 of 4 NPC patients in relapse.

INTRODUCTION

TF, a nonantigenic, nonantibody, low-molecular-weight, dialyzable product of sensitized lymphocytes, has been postulated as a mediator of cellular immunity to a variety of antigens (18). Recently, TF has been applied to the immunotherapy of malignant disease (3, 20, 21, 25, 33). Two postulated as a mediator of cellular immunity to a variety of antigens (18). Recently, TF has been applied to the immunotherapy of malignant disease (3, 20, 21, 25, 33).

Recently, we reported on the treatment of 2 patients with NPC using TF obtained from individuals with a past history of IM (10). The rationale for this form of therapy is that EBV may be an etiological agent in both IM (8, 13, 24, 31) and NPC (6, 14, 16). Lymphocytes from individuals with a past history of IM or normal donors with significant anti-EBV VCA titers may be sensitized to EBV and/or viral-associated antigens and thus may serve as an appropriate source of TF for immunotherapy of NPC.

An in vitro assay of TF activity has been developed in partial support of this hypothesis, the results of which indicate that: (a) in normal EBV-positive subjects a positive correlation exists between humoral immunity to EBV and CMI to NPC; (b) in patients with NPC a positive correlation also exists between the presence of CMI and disease status; (c) both autochthonous and allogeneic reactivities against NPC antigen exist in patients with NPC; (d) TF derived from EBV-positive donors (“EBV-positive TF”) confers in vitro reactivity against NPC antigen to leukocytes of EBV-negative normal individuals and to some NPC patients in relapse; and (e) the inability to convert other NPC patients in relapse to a reactive state sometimes may be due to the presence of serum blocking factor(s).

MATERIALS AND METHODS

Preparation of Soluble NPC Tumor and Normal Tissue Antigen Extracts. A hypotonic (0.7%) NaCl extraction of a single biopsy specimen of NPC was carried out using a modification of the method of Law and Appella (17) to provide a source of soluble tumor antigen. Since normal nasopharyngeal tissue was not available for use as a control antigen, 0.7% NaCl extracts were made in a similar manner from biopsy specimens of normal breast, lung, and prostatic tissue. The protein content of the antigen extracts was determined using the method of Lowry et al. (22), and 0.3-ml aliquots were stored at −20°.

Preparation of TF. TF was prepared using a modification of the technique of Lawrence and Al-Askari (19). Approximately 450 to 500 ml of blood were collected in a sterile Fenwal (JH-1E) plastic bag (Baxter Laboratories, Malton, Ontario, Canada) containing heparin, passed through a Fenwal leukofilter (LP-1 Leuko-Pak) to remove granulocytes, and centrifuged at 2,300 rpm for 5 min in a Sorvall RC-3 centrifuge. The plasma and mononuclear-rich buffy coat were removed with a Travenol extractor into an AE-7 Plasma

Received February 13, 1974; accepted August 7, 1974.
Transfer Set (Baxter Laboratories) and centrifuged at 5,000 rpm for 10 min. The supernatant plasma was removed, the buffy coat in a final volume of 5 to 10 ml was freeze-thawed 7 times, and centrifuged at 20,000 x g for 30 min at 4° in an International Model UV centrifuge, and the supernatant was ultrafiltered through a PM-10 (76 mm) Diaflo membrane (Amicon Corporation, Lexington, Mass.). Each 3 to 5 ml of clear, colorless ultrafiltrate, which represented 1 donor unit of approximately 0.75 to 1.5 x 10^9 mononuclear cells or 2.5 to 5 x 10^9 leukocyte equivalents, was filtered through a Millipore membrane (Millipore Corporation, Bedford, Mass.) and stored at -20°. The ultrafiltrate had a maximal absorbance at 260 nm, and the presence of ribose was determined by the orcinol reaction as described previously (9).

Selection of TF Donors. Normal blood donors were screened through Red Cross blood donor clinics for serum anti-VCA antibody activity using the HR1K subline of Burkitt lymphoma cells in the standard immunofluorescent technique (12).

Titters of 1:16 or greater were arbitrarily defined as positive and titers of less than 1:4 were defined as negative. All individuals with a past history of hepatitis, malaria, or syphilis were excluded. Donors were screened for hepatitis B antigen and positive reactors were rejected. Individuals with titers of less than 1:4 served as “EBV-negative” TF donors and those with titers of 1:16 or greater served as “EBV-positive” donors for in vitro studies.

Peripheral Leukocyte Migration Inhibition Assay. The method of Soborg and Bendixen (32) as modified by Rosenberg and David (29) was used to test selected subjects against the NPC-soluble antigen extract. This assay has been used previously by others to assess CMI to antigens derived from melanoma (4) and breast cancer (1, 4). Four groups of subjects were tested: normal subjects who were either EBV positive (>= 1:16) or EBV-negative (< 1:4) and patients suffering from NPC with their disease in remission or in relapse.

To obtain purified leukocytes, 30 ml of heparinized blood were sedimented using Plasmagel (Laboratoire Roger Bellon, Neuilly, France), the leukocyte-rich supernatant was centrifuged at 1000 rpm for 10 min in a Sorvall GLC-I centrifuge, and the leukocyte pellet was washed consecutively with minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.), a red cell-lysing solution consisting of 0.14 M ammonium chloride—0.017 M Tris buffer (Sigma Chemical Co., St. Louis, Mo.) at pH 7.2 (2), and minimal essential medium, centrifuging after each step at 1000 rpm for 10 min.

The migration of leukocytes from capillaries incubated for 20 hr at 37° in Medium 199 (Grand Island Biological Co.) containing 10% horse serum was determined by projection microscopy, drawing around the periphery of migrating cells and measuring the area by planimetry as described by Maini et al. (23). The area of migration of leukocytes incubated in medium alone was compared to that obtained in medium containing NPC antigen at various concentrations; all determinations were performed in duplicate. Inhibition of migration greater than 20% in the presence of antigen compared to control values in the absence of antigen was considered a positive result.

Assay for TF Activity. After the isolation and washing procedures, leukocytes were divided into equal aliquots and resuspended in 9 ml of Medium 199 containing either 1 ml of the TF preparation to be tested (approximately 1 x 10^8 lymphocyte equivalents) or 1 ml of 0.9% NaCl solution as a control. The cells were incubated for 1 hr at 37° and centrifuged at 1000 rpm for 10 min prior to the assay (29). The migration of TF-treated cells in medium alone was compared to that obtained in medium containing antigen, and these results were compared to the migration of leukocytes incubated in 0.9% NaCl solution, in the presence and absence of antigen.

The effect of TF incubation alone on the migration of leukocytes was also studied.

Statistical Analysis. Data were analyzed for statistical significance using Wilcoxon's nonparametric test (2-tailed) unless otherwise indicated.

RESULTS

Correlation of CMI to NPC Antigen with EBV Antibody Titer. Two groups, each consisting of 10 normal subjects who were either EBV-positive or EBV-negative, were tested with the leukocyte migration inhibition assay against NPC antigen at concentrations of 50, 100, and 175 μg/ml.

All 10 EBV-positive subjects displayed in vitro evidence of CMI to the soluble NPC tumor antigen regardless of concentration (Table 1). The mean migration inhibition was 36.8 ± 3.4% (S.E.) at 50 μg/ml and 48 ± 2.5% at 175 μg/ml, and this difference was significant (p < 0.02).

Conversely, 9 of 10 EBV-negative normal subjects failed to demonstrate migration inhibition at the 50-μg/ml concentration of antigen and the mean migration inhibition was 13.8 ± 5.3% (Table 1). Seven of these subjects were also tested at the higher antigen concentrations of either 100 or 175 μg/ml; 2 subjects tested at 100 μg/ml failed to show migration inhibition and the mean value was 12.5 ± 0.5%. However, 3 of 5 EBV-negative subjects tested at 175 μg/ml demonstrated migration inhibition, and the mean value for the 5 subjects was 19.0 ± 9.5%. The difference in mean migration inhibition between the 10 EBV-positive (36.8 ± 3.4%) and 10 EBV-negative (13.8 ± 5.3%) normal subjects tested at antigen concentrations of 50 μg/ml was significant (p < 0.002). Similarly, the difference in mean migration inhibition between the 8 EBV-positive (48 ± 2.5%) and 5 EBV-negative (19 ± 9.5%) normal subjects tested at 175 μg/ml was also significant (p < 0.02).

Reactivity to Normal Tissue Antigen Extracts. The antigen extracts of normal breast, lung, and prostatic tissue were tested in concentrations ranging from 50 to 360 μg/ml. In over 100 tests involving 50 normal individuals, including 5 of the EBV-positive subjects tested against NPC antigen, and 45 other individuals whose EBV status was unknown, only 1 of the latter subjects reacted. Thus the incidence of nonspecific reactivity was of the order of 2% using this system.

Effect of EBV-positive TF on EBV-negative Leukocytes in Vitro. All 9 EBV-negative normal subjects who failed to demonstrate migration inhibition at the 50-μg/ml concentration of antigen were retested at this concentration after preincubation of their leukocytes with EBV-positive TF.
Table 3. A comparison of the effect of 0.9% NaCl solution and migration inhibition after incubation of their leukocytes with TF incubation in the presence of antigen on leukocytes antigen concentrations of either 50 or 175 pg/ml (Donor 1); derived from an individual with no migration inhibition at antigen using 3 TF preparations: EBV-positive TF (pooled subjects demonstrated migration inhibition (38.7 ± 6.9%) inhibition at 175 pg/ml but not at 50 pg/ml (Donor 2). All 3 and EBV-negative TF derived from a subject with migration of the EBV-negative subjects who demonstrated leukocyte from approximately 25 normal donors), EBV-negative TF incubated with NPC antigen, 50 pg/ml (Table 2). The preincubation mean migration inhibition was 6.0 ± 2.5% while the post-TF incubation value was 27.8 ± 3.9% and this difference was highly significant (p < 0.002). An attempt to convert 1 of the 2 negative responders, exposing TF-treated leukocytes to 175 µg/ml of antigen, was also negative.

No significant difference was observed in the migration of 0.9% NaCl solution or, TF preincubated leukocytes in the absence of antigen (Table 2). Thus, any inhibition of TF-treated leukocytes observed with antigen was not a cumulative effect of nonspecific inhibition by TF plus specific inhibition due to antigen.

Additional evidence that inhibition conferred by TF on the leukocytes of CMI-negative subjects is meaningful is shown in Table 3. A comparison of the effect of 0.9% NaCl solution and TF incubation in the presence of antigen on leukocytes of normal subjects with CMI to NPC antigen was not significantly different. This contrasts with the significant degree of inhibition conferred by TF on the leukocytes of EBV-negative individuals lacking CMI to NPC antigen (Table 2).

Specificity of Transfer. To test for specificity of transfer, 3 of the EBV-negative subjects who demonstrated leukocyte migration inhibition after incubation of their leukocytes with EBV-positive TF were retested at 50 µg/ml concentrations of antigen using 3 TF preparations: EBV-positive TF (pooled from approximately 25 normal donors), EBV-negative TF derived from an individual with no migration inhibition at antigen concentrations of either 50 or 175 µg/ml (Donor 1); and EBV-negative TF derived from a subject with migration inhibition at 175 µg/ml but not at 50 µg/ml (Donor 2). All 3 subjects demonstrated migration inhibition (38.7 ± 6.9%) when their leukocytes were preincubated with EBV-positive TF (Chart 1). None of the 3 demonstrated migration inhibition on incubation of their leukocytes with TF derived from EBV-negative Donor 1, the mean inhibition being 5 ± 1.2%, and only 1 of 3 demonstrated migration inhibition on exposure to TF derived from EBV-negative Donor 2, the mean inhibition being 16.3 ± 5.6% (Chart 1). The conversion of reactivity of the 3 EBV-negative subjects using EBV-positive TF as compared to the results using TF from either EBV-negative Donors 1 or 2 was significant (p < 0.04) using a 1-tailed nonparametric exact test.

Results in Patients with NPC. The NPC patients consisted of 4 patients in remission and 4 patients in relapse when tested. One patient (NPC relapse Patient 1) was included in both remission and relapse categories, being in remission when first tested and in relapse when subsequently tested. All remission patients had received radiotherapy from 3 months to 2 years previously and had no evidence of residual disease when tested. Three of the patients in relapse had recurrence of tumor within 1 year following radiotherapy: Patient 1, who was in remission when first tested, had both local recurrence and distant metastases; Patient 2 had multiple pulmonary metastases in the absence of recurrence in the primary site; and Patient 3 had local recurrence. Patient 4 was studied prior to initial radiotherapy of the primary tumor. All NPC patients had significant EBV antibody titers ranging from 1:16 to 1:1024.

The patients were tested at antigen concentrations of 50 and 175 µg/ml. All remission patients demonstrated migration inhibition at both antigen concentrations (Chart 2); the mean migration inhibition at 50 µg/ml was 28.8 ± 4.0% and that at 175 µg/ml was 39.8 ± 1.4%, but the difference fell short of statistical significance. None of the 4 relapse patients tested at 50 µg/ml and neither of 2 additionally tested at 175 µg/ml demonstrated migration inhibition (Chart 2); the mean

Table 1
Leukocyte migration inhibition assay in EBV-positive and EBV-negative normal subjects
Positive leukocyte migration inhibition is defined as inhibition of migration greater than 20% of that observed in control chambers not containing antigen. All tests on individual subjects were performed in duplicate; area of migration was determined by planimetry (23) and percentage of inhibition was calculated using the formula:

\[ \frac{100 - \text{area of migration in presence of antigen}}{\text{area of migration in absence of antigen}} \times 100 \]

The data were analyzed statistically by Wilcoxon's nonparametric 2-tailed test and the findings are discussed in the text. in before sacrifice.

<table>
<thead>
<tr>
<th>NPC antigen concentration (µg/ml)</th>
<th>EBV positive (&gt;1:16)</th>
<th>EBV negative (&lt;1:4)</th>
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<tbody>
<tr>
<td></td>
<td>No. positive/ no. of subjects</td>
<td>% inhibition</td>
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<td>50</td>
<td>10/10</td>
<td>36.8 ± 3.4&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>175</td>
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<sup>a</sup> Mean ± S.E.
migration inhibition at 50 µg/ml was 4.3% and at 175 µg/ml it was 1.0%. The difference in mean migration at 50 µg/ml between the remission and relapse groups was significant (p < 0.03).

In vitro incubation of leukocytes of Patients 1 and 4 with EBV-positive TF resulted in conversion to a positive response using NPC antigen, 50 µg/ml (Chart 2). Similar testing at 175 µg/ml failed to convert the leukocytes of Patients 2 and 3 after TF incubation.

To determine whether blocking factors might be present in the serum of these relapse patients and possibly influence the response to TF, leukocytes were washed in an attempt to remove any "factor(s)" that might interfere with reactivity. Washing of the leukocytes of Patient 1, who responded to TF incubation, failed to establish reactivity to NPC antigen, 50 µg/ml. Conversely, in Patients 2 and 3, who failed to respond to TF incubation, leukocyte washing established reactivity. However, in Patient 4, who responded to TF incubation,
Transfer of Cellular Immunity against NPC

EBV NEG. RECIPIENT IRH OR DR

Chart 1. Comparison of effect of TF from EBV-positive (POS.) and EBV-negative (NEG.) normal donors on leukocyte migration inhibition against NPC antigen, 50 µg/ml, in 3 EBV-negative normal recipients in vitro. EBV-negative TF (Donor 1) was derived from an individual with no migration inhibition at NPC antigen concentrations of either 50 or 175 µg/ml; EBV-negative TF (Donor 2) was derived from a subject with migration inhibition at 175 µg/ml but not at 50 µg/ml.

EBV-positive subjects had a previous history of IM and all had anti-VCA antibody titers of 1:64. The remaining 7 subjects had titers ranging from 1:16 to 1:256.

Group 2 consists of 4 EBV-negative subjects who were CMI negative at all concentrations of antigen.

The 3rd group consists of 3 EBV-negative individuals who did not display CMI at the 50 µg/ml concentration of NPC tumor antigen but who did react at a concentration of 175 µg/ml; 2 of the 3 subsequently displayed CMI at the 50 µg/ml concentration when their leukocytes were preincubated with EBV-positive TF.

Three other EBV-negative normal subjects were tested only at the antigen concentration of 50 µg/ml; 2 were negative and would be included either in Group 2 or Group 3. The 3rd subject was CMI positive and was the sole responder to 50 µg/ml in the entire EBV-negative group. None of the 10 EBV-negative subjects in Groups 2 and 3 had a history of IM, which was consistent with the previous experience of others (8, 24).

It is not possible to state whether the difference in reactivity between EBV-negative Groups 2 and 3 is qualitative or quantitative. A quantitative analogy to tuberculin sensitivity may exist in that some individuals are nonreactive to 1 or 5 units of tuberculin purified protein derivative, but are reactive to 250 units. Similarly, EBV-negative subjects who were reactive only to higher concentrations of tumor antigen may be mildly sensitized to EBV, while EBV-negative subjects who failed to inhibit at all antigen concentrations may be completely unreactive. If true, this would suggest that the correlation between humoral immunity to EBV and cellular immunity to NPC in normal subjects may be imperfect.

Support for the specificity of transfer is derived from the experiments showing that while EBV-positive TF converted the

leukocyte washing also established CMI reactivity (Chart 3).

DISCUSSION

A positive correlation has been demonstrated between CMI to a soluble tumor extract of NPC and humoral immunity to EB virus in normal individuals. The normal subjects can be divided into 3 main groups. Group 1 consists of the 10 EBV-positive subjects, all of whom demonstrated CMI to the tumor antigen at all concentrations tested. Only 3 of these 10

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leukocytes of EBV-negative recipients to reactivity in vitro in 3 of 3 subjects, EBV-negative TF from Donor 1 (CMI negative at all concentrations) consistently failed to do so, while EBV negative TF from Donor 2 (CMI positive at high concentrations but CMI negative at low concentrations) failed to convert 2 of 3 individuals. Thus the reactivity to NPC antigen conveyed by the TF would seem to be specific and correlate with the presence of humoral immunity to EBV in the TF donor.

A positive correlation was also noted between the presence of CMI against NPC antigen and disease status in patients with NPC; those in remission consistently demonstrated positive reactivity and those in relapse did not. One of the 4 remission patients who demonstrated migration inhibition provided the source of NPC antigen for all tests performed, thus demonstrating autochthonous reactivity in this system.

The ability to convert the leukocytes of NPC relapse Patient 1 to reactivity with EBV-positive TF coincides with the inability to demonstrate blocking factors, whereas the inability to convert the leukocytes of Patients 2 and 3 to reactivity with TF corresponds with the demonstration of serum blocking activity by the washing procedure (Chart 3). However, the ability to convert the leukocytes of Patient 4 to reactivity both by the washing procedure and by TF incubation suggests that the interrelationship between apparent blocking factors and the response to TF is variable. The establishment of reactivity after extended washing of leukocytes is in agreement with the results of Currie and Basham (5) and suggests the possibility that "blocking factors" may be present in the sera of some patients with active disease (30) and may in some but not all cases block the ability of TF to effect conversion. If TF can on occasion overcome blocking activity, as might be the case in Patient 4, this would increase its potential value as an immunotherapeutic agent.

Since tumor-specific antigens cannot be purified at present, it is difficult to be certain that any of the subjects were in fact reacting against these antigens. However, the autochthonous reactivity in the tumor donor and the short duration of incubation (20 hr) in the other subjects virtually rules out an allogeneic response (15). None of the normal subjects or patients who had blood transfusions that might give rise to allotypical sensitization and nonspecific reactivity. This study does not exclude the possibility that reactivity against the presence of EBV alone in the preparation was being measured. However, the presence of particles identical to EBV are rarely, if ever, seen in electron microscopic studies of fresh tissue and arise mainly in lymphoblastoid cells derived from tissue culture (7).

Recently, using an anticomplementary fluorescent staining method to examine biopsy sections of NPC, zur Hausen (35) demonstrated a clearly positive fluorescence against the epithelial cell component of NPC tissue using EBV antibody-positive sera from normal donors. The fluorescence depended on the concurrent presence in the test sera of antibodies against nonstructural EBV-associated membrane antigens. No fluorescence against EBV early antigen or structural antigens (VCA) was detected. In addition, in hybridization studies, zur Hausen and Schulte-Holthausen (36) have detected evidence for the presence of the EBV genome in the epithelial cell component. It would be of interest to determine whether the humoral immunity detected by zur Hausen correlates with the CMI against NPC antigen detected by the peripheral leukocyte migration inhibition assay.

The presence of cross-reactivity against NPC antigen not only in EBV-positive normal subjects but also in NPC patients suggests an immune response against an oncogenic virus, consistent with previous findings for oncogenic viruses known in animals (26) and suspected in man (11). This finding may provide an additional, although indirect link, between EBV and NPC; however, an oncogenic or merely passenger role for EBV remains to be determined.

The findings also indicate that the peripheral leukocyte migration inhibition assay may be adapted as an in vitro assay for TF activity. Recently, Utermohlen and Zabriskie (34) have shown that measles-specific TF converted nonsensitized leukocytes in patients with multiple sclerosis to a reactive state as measured by the leukocyte migration assay. These results are in contrast to those of Paque et al. (27), who concluded that, although nondialyzable TF converted leukocytes to antigen responsiveness in vitro in the classical indirect macrophage MIF assay, dialyzable TF was inactive. However, in this study, TF was prepared in a manner different from that of Paque, and in addition there is now evidence that the classical MIF assay does not measure the same lymphokine as does the peripheral leukocyte migration inhibition assay (28). The factor detected by the leukocyte migration inhibition assay is called leukocyte inhibition factor and has been shown by Rocklin to be distinct from MIF. Leukocyte inhibition factor is released by specifically sensitized lymphocytes in the presence of antigen and exerts its inhibitory effect on polymorphonuclear leukocytes.

LoBuglio et al. (21) have reported that in vivo administration of dialyzable TF from a related donor with tumor-specific immunity, as measured by MIF production in vitro to sarcoma antigen, resulted in MIF production in vitro by the tumor-bearing recipient.

Our results indicate that the peripheral leukocyte migration inhibition assay may be useful for in vitro measurement of tumor immunity against NPC, and when adapted as an assay for TF activity it may be predictive of which donors might provide a suitable source of TF and which patients might benefit from TF therapy in vivo.

ACKNOWLEDGMENTS

We thank Dr. J. M. Bowman and Catherine Anderson (Canadian Red Cross Blood Bank, Winnipeg, Manitoba, Canada) for the preparation of buffy coat; Dr. J. C. Wilt, Dr. A. M. Wallbank, R. Peeling, and E. McGuire for determination of anti-EBV antibody titers; J. McLennan for determination of antigen protein concentration; Dr. G. A. Brown and Dr. R. K. Watson for providing biopsy material; Dr. G. R. Hogg for reviewing histological sections; Dr. N. Nelson for assistance with the statistical analysis and D. Faulkner for typing the manuscript.

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Transfer of Cellular Immunity against NPC


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Cancer Res 1974;34:3095-3101.

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