AN IMMUNOLOGIC BASIS FOR DETECTION OF OCCULT PRIMARY MALIGNANCIES OF THE HEAD AND NECK

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After extensive evaluation of patients with metastatic neck disease and clinically undetectable primary cancer of the head and neck, the clinician is often faced with the difficult question of subsequent management. In this study, sera from 11 patients with clinically occult carcinoma and metastatic lymphadenopathy were studied for Epstein-Barr virus-associated antigens. These were compared with 35 sera from patients with known nasopharyngeal carcinoma at all stages of disease and treatment and with 212 sera from control patients with other head and neck tumors, patients with lymphoma, and normal controls. There was a significant correlation between high antibody titers to Epstein-Barr virus, especially in the serum IgA fraction, and the presence of nasopharyngeal carcinoma. Thus, identification of occult nasopharyngeal carcinoma by immunologic means may have important application in the selective management of the patient with an unknown head and neck primary malignancy.


The patient with a painless neck mass must be regarded as having a malignancy until it is proven otherwise. Usually, thorough examination of the head and neck skin and mucosal surfaces by a clinician skilled in the techniques of examination of the upper airway will reveal the primary malignancy. Occasionally, despite exhaustive history, physical examination, and special investigations, including roentgenologic and endoscopic studies and biopsies of suspect sites in the head and neck, the source of the malignancy is not detected. These patients with metastatic cervical disease and occult primary cancer represent a small but significant proportion of patients presenting with head and neck cancer. The incidence of occult primary malignancies in studies of metastatic cancer of the neck is 3 to 9%. The majority of these lesions are squamous cell carcinomas. Follow-up and autopsy studies reveal that the commonest site of origin today is the nasopharynx. Thus, a means of detecting occult or subclinical primary nasopharyngeal carcinoma (NPC) would be of value in the diagnosis and treatment of patients with metastatic neck disease and occult primary cancer.

There is a significant relationship between NPC and Epstein-Barr virus (EBV) which was first observed by Old et al. Subsequent reports have demonstrated the presence of high titers of antibodies to EBV antigens in the sera of NPC patients throughout the world. The specificity of these EBV-associated antigens is such that high antibody titers to the viral capsid antigen (VCA), early antigen (EA), and the EBV antigens in the serum IgA fraction in particular are usually associated with NPC. High titers of EBV-specific IgA serum antibodies and antibodies to EA are uncommon in patients with neoplasms in other regions of the head and neck and in the normal population. When these anti-EBV antibodies are detected in patients who do not have NPC, their titers are generally much lower than those seen in patients with NPC. In a study of EBV-specific IgA serum antibodies in non-American patients with NPC, these antibodies were present, often at high titers, in 93% of the untreated patients; less than 5% of 73 patients with other untreated head and
neck cancers and normal controls had VCA-specific IgA in their sera. Thus the detection of high antibody titers to EBV-associated antigens in the sera of patients with metastatic neck disease with clinically occult primaries could be of value in guiding the direction of further diagnostic testing toward a search for occult NPC.

It was the purpose of this work to determine in American patients whether or not EBV-associated antigens are specific for NPC and to study the sera of patients with occult head and neck primaries for the presence of these EBV-associated antibodies.

**Materials and Methods**

**Patients**

To establish specificity of EBV-associated antigens in American patients, 35 sera from patients with NPC, both untreated and treated, and at all stages of disease, were studied for the presence of antibodies to EBV-associated antigens. For comparison, 87 sera from patients with other head and neck cancers, 80 sera from patients with lymphomas, and 47 sera from control patients with no cancer were also tested for antibodies to EBV antigens.

Eleven patients who had occult primary malignancy and cervical metastases and were seen at the Mayo Clinic were studied for the presence of antibodies to EBV-associated antigens. These 11 patients fulfilled the criteria for the diagnosis of the unknown primary as described by Comess et al.; namely 1) no history of previous malignancy or of surgical ablation of any indeterminate lesion, 2) no history of definite symptoms related to a specific organ, 3) no clinical or laboratory evidence of a primary neoplasm, proven or not, and 4) the presence of one or more cervical masses proved histologically to be cancer.

The 11 patients with occult primary malignancy had been treated or followed without specific treatment or they were new patients.

We obtained coded vials of sera from a group of 38 patients (International Agency for Research on Cancer [IARC], Lyons, France) who had stage I and II NPC (WHO classification), or other head and neck cancer, and normal controls. Indirect immunofluorescent antibody titers to EBV antigens were determined and the sera, based on these results, were divided into two categories: "most probably NPC" or "unlikely NPC."

**EBV-Specific Immunofluorescence Tests**

The sera from all patients were tested by the VCA and EA test and for VCA antibody to EBV antigens in the serum IgA fraction. These tests are all indirect immunofluorescent tests.

**VCA test:** The VCA test as described by Henle and Henle detects intracellular structural antigens (viral capsid antigens) in EBV-producing cell lines. We used the virus-producing P3HR1K cell line, standard stock slides containing antigen prepared from these cells, and immunofluorescent reagents containing goat antibodies to human IgG (Hyland, Costa Mesa, California). Test sera were titrated for VCA antibody levels. The usual dilutions of the test sera were fourfold (1:10, 1:40, 1:160, 1:640). The slides were examined under an American Optical fluorescent microscope with a vertical light source. Antibody titers in the IgG serum fraction were determined and a positive titer (1:10) merely indicates that the individual has been infected with EBV at some stage in his life.

**EA test:** The EA test detects early antigens produced within hours after superinfection by EBV of a nonvirus-producing lymphoblastoid line such as Raji. Early antigen synthesis does not require DNA synthesis and appears to consist of two different antigens—a diffuse (D) and a restricted (R) component.

Stock slides of Raji cells experimentally infected with EBV were prepared and the test sera were added in appropriate dilutions. As in the VCA test, goat antihuman IgG was used as the fluorescent reagent and the slides were examined under the fluorescent microscope with a vertical light source for determination of the antibody titer, by establishing the last serum dilution to give a fluorescent reaction. Antibodies to EA reflect an active infection with the virus, and therefore high titers are found in patients with infectious mononucleosis, Burkitt’s lymphoma, and NPC.

**EBV antibodies in serum IgA fraction:**

The IgA antibodies to the EBV antigens can be determined by using fluorescent goat antihuman IgA (Hyland, Costa Mesa, California) as the fluorescent reagent as a substitute for the antihuman IgG that is used in the VCA and EA tests. In this case, only VCA-associated antibody titers in the serum IgA fraction were determined.

**Results**

**Specificity of Tests for EBV in American NPC**

All the 35 sera from patients with NPC had positive titers for VCA, as opposed to lower percentages in the control groups. Seventy-seven
percent of the 47 normal sera had positive titers for VCA, whereas 91% of the sera from patients with other head and neck cancers and 88% of the sera from lymphoma patients had positive titers. Geometric mean titers were performed on these sera, and the differences in results between the patient groups were significant (p < 0.001) by chi-square analysis (Table 1). In addition, a fourfold difference in titers is considered significant in EBV studies.

Although 94% of the NPC sera had positive EA titers, fewer control patients' sera had positive EA titers as compared with VCA titers. Statistical evaluation comparing the titers of the NPC sera with those in the three control groups showed highly significant differences (p < .001; Table 2).

Serum antibodies to EBV-induced VCA in the IgA immunoglobulin fraction showed the highest specificity for patients with NPC. All but two of the sera from American patients with untreated NPC showed positive IgA titers to EBV antigens. Six of the 16 sera from patients who were in clinical remission without evidence of recurrent disease had negative titers, whereas the remaining 10 sera had antibodies detectable at low levels (geometric mean titer, 6.50). By contrast, none of the three control groups had more than 11% of sera with positive IgA anti-VCA titers (Table 3). By statistical evaluation, the differences in titers between the NPC group and the three control groups were highly significant (p < .001).

### Results of Immunofluorescent Testing for Coded Sera

Immunofluorescent tests were performed on the 38 coded sera sent from the IARC. EA titers were plotted against IgA titers to VCA as shown in Fig. 1. Ten patients were classified as “most probably NPC” whereas 28 patients were classified as “unlikely NPC.” The criteria used as the dividing line between control and NPC sera were chosen on the basis of previous work. An EA titer of 40 or more and an IgA antibody titer to VCA of 10 or more were considered consistent with a diagnosis of “most probably NPC.”

After the code was broken, we noted that we had effectively placed 37 of the 38 coded sera into their correct categories on the basis of the results of the EA and IgA antibody tests. All of the 27 control patients fell within the area below a line drawn at an EA titer of 40 intersecting with the IgA antibody titer of 10. All but 1 of the 11 NPC patients had titers that were outside this area. Thus it would appear that EA and EBV IgA antibody levels can be used to differentiate reliably and consistently early-stage NPC from control sera.

### Tests for Antibodies to EBV Antigens in Patients With Occult Primaries

Eleven patients, four of whom were untreated patients and seven of whom were previously treated patients with cervical metastasis and occult primary malignancy, were seen at the Mayo Clinic during the period of this study. The immunofluorescence test battery of VCA, EA, and EBV IgA antibodies was performed on
Fig. 1. Early antigen (EA) antibody titers versus IgA antibody titers to Epstein-Barr virus (EBV) in 38 coded sera from patients with NPC and controls.

the sera from these patients. These results, together with clinical data for each patient, are shown in Table 4. A scattergram illustrating the distribution of these sera by EA versus IgA antibodies to EBV is shown in Fig. 2. Only one of the sera (no. 2) fell into the range of “most probably NPC,” although one patient (no. 7) was at the marginal zone between the two areas. A pathologist (L.H.W.) reviewed the biopsy tissue that was removed from the neck of each patient. Four patients (nos. 2, 7, 8, and 9) had histopathologic features consistent with an NPC origin, whereas the seven remaining patients showed histologic patterns distinctly different from these. Two of the four patients (nos. 8 and 9) with histopathologic features consistent with NPC had been successfully treated with radiation therapy with no clinical evidence of recurrence 1 year later.

DISCUSSION

Historically, Virchow in 1849 provided the first description of cervical lymphadenopathy as a feature of the metastatic spread of visceral tumors. This was further elaborated on by Troisier and Crile, who emphasized the spread-limiting effect of the lymph glands and their accessibility for radical excision. Martin and Romieu in 1952 summarized the problem of cervical metastatic disease succinctly when they stated, “Asymmetric enlargement of one or more cervical lymph nodes . . . usually is due to metastasis from a primary lesion in the mouth or pharynx.”

In the first half of this century, metastasis to the cervical lymph nodes from an occult source was most commonly found to be due to thyroid cancer. With the advent of better fresh-frozen histopathologic techniques and radioisotope studies, the incidence of occult thyroid neoplasms with cervical metastases has decreased quite markedly. Today, the nasopharynx is the most frequent site of these occult primary malignancies with cervical metastases which subsequently manifest themselves clinically (or become determinate cases).

It was the occult NPC to which we addressed our attention in this study in an attempt to
<table>
<thead>
<tr>
<th>Pt no.</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Date of diagnosis</th>
<th>Node biopsy</th>
<th>Treatment</th>
<th>Current status</th>
<th>Antibody titers</th>
<th>Other</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>46</td>
<td>1977</td>
<td>Grade 2 squamous cell carcinoma</td>
<td>Split-course radiation therapy to head and neck, 4,500 rads</td>
<td>Alive, no primary found</td>
<td>160</td>
<td>10</td>
<td>&lt;5</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>...</td>
<td>1976</td>
<td>Suggestive of lymphoepithelioma</td>
<td>Nil specific</td>
<td>Alive, no primary found</td>
<td>160</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>63</td>
<td>1975</td>
<td>Grade 2 squamous cell carcinoma. One involved upper deep jugular node.</td>
<td>Neck dissection. Pharyngeal biopsies.</td>
<td>Alive, no primary found</td>
<td>60</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>52</td>
<td>1976</td>
<td>Grade 4 undifferentiated carcinoma. Adenocarcinoma by electron microscopy.</td>
<td>Lymph node biopsy. Nasopharyngeal biopsy</td>
<td>Alive, no primary found</td>
<td>10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>74</td>
<td>1974</td>
<td>Grade 4 undifferentiated carcinoma. Suspect oral cavity origin.</td>
<td>Nil specific</td>
<td>Alive, no primary found</td>
<td>80</td>
<td>10</td>
<td>&lt;5</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>61</td>
<td>1968</td>
<td>Metastatic grade 4 squamous cell carcinoma</td>
<td>Node biopsy only</td>
<td>Alive, no primary found</td>
<td>40</td>
<td>&lt;10</td>
<td>&lt;5</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>47</td>
<td>Dec 1973</td>
<td>Grade 3 squamous cell carcinoma</td>
<td>5,000 rads Dec 1973. Total parotidectomy and neck dissection March 1974; 10 involved lymph nodes in neck specimen.</td>
<td>Died, carcinomatosis, Sept. 1974</td>
<td>160</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>70</td>
<td>1975</td>
<td>Grade 3 nonkeratinizing squamous cell carcinoma. Possible nasopharynx origin.</td>
<td>Suprathyroid dissection. Radiation therapy, 5,000 rads.</td>
<td>Alive, no primary found</td>
<td>160</td>
<td>20</td>
<td>&lt;5</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>58</td>
<td>1975</td>
<td>Grade 4 squamous cell carcinoma. Possible nasopharynx origin.</td>
<td>Parotidectomy with postoperative radiation, 5,580 rads.</td>
<td>Alive, no evidence of recurrence</td>
<td>640</td>
<td>10</td>
<td>&lt;5</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>67</td>
<td>1976</td>
<td>High-grade undifferentiated carcinoma. Possible renal or respiratory origin.</td>
<td>Total parotidectomy and neck dissection. Radiation therapy postop.</td>
<td>Alive, no evidence of recurrence</td>
<td>80</td>
<td>10</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

* EBV = Epstein-Barr virus; VCA = viral capsid antigen; EA = early antigen; EBNA = EBV-nuclear antigen.
differentiate serologically the occult NPC from other carcinomas in the head and neck. The first question, that of specificity of the EBV antigens in American patients with NPC, was answered when we showed conclusively that all three EBV-associated antigens studied were present in these patients, and that high antibody titers were present to these antigens in all the untreated patients with NPC. In particular, the differences in geometric mean titers between the control groups and the American NPC patients were significant, especially in the case of the anti-EA and anti-VCA (IgA) antibodies.

The question of whether application of the test to detect occult NPC was feasible was directed to 38 coded sera from patients with known pathology as well as normal controls. By the criterion of a positive antibody titer to EA of 40 and to IgA of 10 as the dividing line between those with a high probability of being NPC and those unlikely to be NPC, 37 of 38 test sera had been categorized correctly when the code was broken. The majority of the control sera had no antibodies to VCA in the serum IgA fraction, whereas none of the control sera had values outside the "control" area beneath the line. The serum from the patient with stage I NPC with EBV antibody titers similar to those of the control groups is being rechecked to ascertain that it was not from a treated patient, in which case one might expect, after successful radiation therapy, a level of EA and IgA antibodies similar to that in the controls.

The final question was whether we could apply the criteria described above to sera from treated and untreated patients presenting with occult primaries. Of the 11 patients who presented with this diagnosis and from whom sera were available for study, 4 were untreated patients. One of these four (no. 2) had titers that were consistent with the diagnosis of NPC and histopathologically compatible with a nasopharyngeal primary. This patient was first examined in 1972 and she received no treatment after surgical removal of the node. A serum sample from this patient was examined for antibodies to EBV antigens in 1975, with the results and conclusions noted above. Recently, she returned with a second enlarged cervical node. Biopsy of the nasopharynx this time revealed the
presence of NPC. Thus the value of EBV serology in the diagnosis of a nasopharyngeal primary was supported in this case.

One patient (no. 7) in the treated group of patients with unknown primaries had a grade 3 squamous cell carcinoma with 10 involved lymph nodes; despite surgery and irradiation to the head and neck, the patient died of carcinoma 10 months after histopathologic diagnosis. This patient's antibody titers of 40 for EA and 10 for EBV IgA were at the dividing line between the control and "probably NPC" groups.

The remainder of the treated patients had antibody titers well within the "control" area. Two of these patients (nos. 8 and 9) had histopathologic features consistent with an NPC primary and had been successfully irradiated. Unfortunately, no pretreatment sera were available from these patients.

Since it has been established that anti-EBV antibody titers tend to decrease to low or undetectable levels in successfully treated NPC patients, it is not possible to reach any conclusions on the significance of EBV serology in these two patients.

More recently, EBV serology was useful in the identification of a nasopharyngeal primary in another patient who presented with cervical metastasis. The histopathology of the involved nodes was suggestive of NPC, but no cancer was found in tissue taken from the nasopharyngeal area, although the EBV serology also pointed to a nasopharyngeal primary (VCA titer was 320, EA titer 80, and IgA titer 40). Based on this information, tissue was again removed from the nasopharynx, and this time tumor was identified in the specimens. Thus the value of EBV serology as an adjunct to pathology in the identification of primary tumors originating in the nasopharynx was further substantiated in this patient. Of these EBV parameters, the EBV IgA antibody titer appears to be the most specific and reliable marker for NPC. Obviously, it is important to try to substantiate these preliminary findings with a larger group of patients.

In summary, we believe that EBV serology is a valuable parameter to be used in the evaluation and clinical management of patients with occult head and neck primary tumors with cervical metastases to delineate those with occult NPC.

REFERENCES