Immunologic Studies in Pernicious Anemia

By Chiaki Tai and James E. McGuigan

Many investigators have described antibodies to intrinsic factor (IF) in sera from patients with pernicious anemia.1-4 These antibodies have been classified as "blocking" and "binding" antibodies. Antibodies to IF designated as blocking antibodies are those which combine with IF inhibiting subsequent complex formation between vitamin B₁₂ and intrinsic factor. Binding antibodies are those which bind intrinsic factor molecules before or following the complexing of intrinsic factor with vitamin B₁₂ but do not prevent formation of intrinsic factor-vitamin B₁₂ complexes. In addition to antibodies to intrinsic factor there are in the sera of approximately 90 per cent of patients with pernicious anemia antibodies to parietal cell cytoplasmic constituents5-7 which have been detected by complement fixation5-8 and immunofluorescent techniques.5,7

Detection of intrinsic factor and parietal cell antibodies, in addition to certain clinical and morphologic findings in pernicious anemia, has raised the question whether pernicious anemia is an autoimmune disease, i.e., a disease in which immunologic phenomena play an integral role in either the initiation or perpetuation (or both) of pathologic events in the disease process. To date there is no direct evidence to support the speculation that antibodies to intrinsic factor play an etiologic role in the pathogenesis of the gastric atrophy which characterizes classical adult Addisonian pernicious anemia. In 30 per cent to 50 per cent of patients with pernicious anemia, antibodies to intrinsic factor cannot be demonstrated with currently available methods. There has been no correlation established between the absence, presence, or levels of antibodies to intrinsic factor and the clinical course of patients with pernicious anemia.8,9 Furthermore to date there is no convincing evidence that circulating antibodies damage normal tissue components in the absence of lymphocyte sensitization.10,11

In studies of experimental autoimmune disease passive transfer of the immunologic disease has been accomplished by transfer of sensitized lympho-
cytes\textsuperscript{12} but not by transfer of antibodies; delayed type hypersensitivity is believed to play the crucial role in the induction of a variety of autoimmune states.

We have examined the lymphocyte transformation\textsuperscript{13-17} characteristics of patients with pernicious anemia in order to identify a potential role for cellular immunity in pernicious anemia. When small lymphocytes in culture are exposed to an antigen to which they are immunologically sensitized or when they are exposed to a nonspecific mitogen such as phytohemagglutinin (PHA)\textsuperscript{17} they undergo morphologic and functional changes designated as lymphocyte transformation. The lymphocytes increase in size, the nuclei become more esinophilic, the cytoplasm more basophilic. Prominent nucleoli appear. Vacuoles are seen in the cytoplasm adjacent to the nucleus. In association with these morphologic changes there is increased synthesis of RNA and protein and increased uptake of tritiated thymidine into DNA. In experimental studies lymphocyte transformation demonstrates a high degree of correlation with delayed (cell mediated) sensitivity.\textsuperscript{18}

\textbf{Materials and Methods}

\textit{Patients}

A group of twenty-nine patients with classic Addisonian pernicious anemia with the diagnosis established by customary clinical and laboratory criteria were evaluated in this study. The patients included 7 males and 22 females, from 39 to 88 years in age. All patients had a clinical history of pernicious anemia exceeding three years in duration. All patients were being treated with monthly intramuscular injections of vitamin B\textsubscript{12}. Eighteen hospitalized patients of similar age distribution to the patients with pernicious anemia were utilized as control subjects.

\textit{Antibody Studies}

\textit{Determination of Autoantibodies in the Sera of Patients with Pernicious Anemia:} 1) Blocking antibodies to intrinsic factor. The presence of blocking antibodies to intrinsic factor was determined by use of the charcoal-assay technic.\textsuperscript{18} Blocking antibodies to intrinsic factor were considered to be present when 0.1 ml of the patient's serum reduced by more than 50 per cent the binding of 2 ng of \textsuperscript{57}Co-cyanocobalamin by intrinsic factor contained in human gastric juice. 2) Binding antibodies to intrinsic factor. Binding antibodies to intrinsic factor were determined utilizing the radioimmunodiffusion technic as modified from Samloff et al.\textsuperscript{4} 3) Antibodies to parietal cell antigen. The microsomal parietal cell antigen was purified from human gastric mucosa by use of the ficin method as described by Baur et al.\textsuperscript{19} The presence of antibodies to the microsomal parietal cell antigen was determined by using complement fixation technics using fresh sheep red blood cells.\textsuperscript{20}

\textit{Lymphocyte Culture and Transformation Studies}

\textit{Antigen preparations used in lymphocyte cultures.} 1. Human gastric juice (homologous) was collected following subcutaneous administration of 50 mg of Histalog (Lilly) by aspiration with a gastric tube after an overnight fast. Gastric juice was collected by manual aspiration and immediately placed into an ice bath. To inactivate pepsin activity the gastric juice was adjusted promptly to pH 10 by addition of 1 M sodium hydroxide. The gastric juice was maintained at pH 10 for 30 minutes on ice and then adjusted to pH 7 by addition of 1 M hydrochloric acid. Pooled neutralized gastric juice was then extensively dialyzed for 48 hours at 4 C against 0.15 NaCl-0.01 M potassium phosphate, pH 7.4 (phosphate buffered saline). Intrinsic factor content was measured by the charcoal assay technic\textsuperscript{18} and the protein content was estimated by optical density determinations using a Beckman
DU-2 Spectrophotometer at 280 mÅ (standardized with bovine serum albumin: E 1% 1 cm. = 6). The IF binding capacity of the gastric juice preparation was 61 ng. vitamin B\(_{12}\) per ml. 2. Highly (but not completely) purified intrinsic factor for use as antigen was prepared from human gastric juice according to the method of Chosy and Schilling\(^2\) using a series of column fractionation procedures. The IF binding capacity of these preparations were 300 to 500 ng vitamin B\(_{12}\) per ml. 3. Normal human gastric mucosa was prepared from surgically resected human stomach kept at -20 C. until use. The mucosa following separation from the underlying submucosa was minced in 0.15 M NaCl and then rapidly frozen and thawed ten times by alternate rapid passage from a 37 C. water bath to a bath containing dry ice and acetone. The resultant freeze-thawed homogenate was centrifuged at 700 × g. for 10 minutes. The protein content of the supernatant solution was adjusted with 0.15 M NaCl to 250 µg. per ml. for use: the IF binding capacity was 30 ng vitamin B\(_{12}\) per ml. The antigens to which the cultured lymphocytes were exposed were sterilized by filtration through millipore filters (N-0.4 µ). Following preparation of antigens they were maintained at -20 C. until used.

**Lymphocyte preparation and culture.** Relatively pure preparations of lymphocytes (85 to 90 per cent) were prepared from 20–40 ml. of heparinized peripheral venous blood by use of the method of Hasting and his associates.\(^{13}\) The lymphocytes were then washed twice with Hank’s balanced salt solution. The lymphocytes were cultured in Eagle’s minimum essential medium containing 20 per cent calf serum (inactivated by heating at 56 C. for 30 minutes), 1-glutamine 30 µg., penicillin G 200 units and potassium dihydrostreptomycin 0.2 µg. per ml. Two ml. of the lymphocyte preparation, at a concentration of 2 × 10\(^6\) cells per ml., were placed in each 15 × 1.6 cm. sterile disposable polyethylene culture tube. Cultures were performed for 96 hr. at 37 C.

**Lymphocyte transformation.** Lymphocyte transformation was determined by measurement of the uptake of \(^{3}H\)-thymidine into DNA. Five hours prior to the termination of the culture period 2 µc. of \(^{3}H\)-thymidine (specific activity 6.7 C per mM, New England Nuclear Corporation, Boston, Mass.) were added to each tube. At the completion of the culture period, the culture tubes were placed in an ice bucket and 2 ml. of 5 per cent trichloracetic acid, maintained at 4 C., were added with mixing to each tube. The tubes were then centrifuged at 4 C. at 700 × g. for 20 minutes. The supernatant solutions were removed and discarded and the procedure was repeated. The resultant precipitates were then washed with cold absolute methanol. Centrifugation was repeated. The precipitant was solubilized by addition of 0.5 ml. Hyamine hydroxide followed by incubation for 20 minutes at 65 C. The solutions were transferred to 15 ml. of Bray’s solution\(^2\) and radioactivity was measured using a Packar Tri-Carb Scintillation Spectrometer. Quantitative estimation of lymphocyte transformation was determined by measurement of uptake of tritiated thymidine into DNA as described by Dutton and Eady.\(^{14}\) The presence and magnitude of lymphocyte transformation were evaluated by determining the ratio of \(^{3}H\)-thymidine uptake in experimental samples compared with that of control tubes to which no antigen or PHA had been added (negative control). A ratio (E/C; E = experimental, C = negative control) greater than 3 was interpreted as a positive response indicative of the presence of lymphocyte transformation.

**Series A.** Lymphocyte cultures from 16 patients with pernicious anemia and 18 control subjects were incubated in the presence of each of the following: 1) 0.1 ml. human gastric juice, 2) 0.1 ml. gastric mucosal homogenate, 3) 0.1 ml. of intrinsic factor preparation, 4) 0.1 ml. PHA-P (1:10).

**Series B.** Long-term cultures (7 days) were performed with lymphocyte cultures from five normal individuals and lymphocyte cultures from 16 patients with pernicious anemia. In addition to positive (PHA-containing) and negative controls the lymphocytes were cultured with 0.2 ml. of a concentrated gastric juice preparation with an I.F. vitamin B\(_{12}\) binding capacity of 180 ng./ml. In order to assure lymphocyte viability through the extended period of observation the culture medium was replaced with fresh culture medium three days and five days following the initiation of the culture period.
RESULTS

Estimation of Circulating Antibodies

1. Blocking antibodies to intrinsic factor. Antibodies which blocked the formation of $[^{57}\text{Co}]$-cyanocobalamin-intrinsic factor complexes were found in the serum of 19 to 29 of the patients with pernicious anemia and in none of the control sera.

2. Binding antibodies to intrinsic factor. Fifteen of the 29 patients with pernicious anemia and none of the control subjects were found to have binding antibodies to intrinsic factor in their sera.

3. Parietal cell antibodies. Of 29 patients with pernicious anemia 21 were found to have complement fixing antibodies to the parietal cell antigen preparation.

Lymphocyte Transformation

Series A. Lymphocyte cultures from 3 of 14 patients with pernicious anemia exhibited lymphocyte transformation when cultured in the presence of purified intrinsic factor (0.1 ml. with a vitamin B$_{12}$ binding capacity of 300 ng./ml.). Lymphocyte transformation was not observed in any of the lymphocyte cultures from control subjects cultured with this intrinsic factor preparation.

Lymphocyte cultures from 2 of 29 pernicious anemia patients underwent transformation when 0.1 ml. of homologous gastric juice (IF vitamin B$_{12}$ binding capacity 61 ng./ml.) was included in the culture (Table 1). Both of these patients were among the three patients whose lymphocytes transformed when cultured with the purified intrinsic factor preparation (above). Lymphocyte transformation was determined to be present when the ratio of uptake of tritiated thymidine into DNA in the experimental cultures as compared with that of the negative cultures was greater than 3.0.

<table>
<thead>
<tr>
<th>Patient</th>
<th>cpm Negative Control</th>
<th>cpm PHA 1:10</th>
<th>cpm Gastric Mucosal Homogenate</th>
<th>cpm with Gastric Juice</th>
</tr>
</thead>
<tbody>
<tr>
<td>KI</td>
<td>151</td>
<td>51344</td>
<td>340*</td>
<td>290</td>
</tr>
<tr>
<td>ES</td>
<td>164</td>
<td>1849</td>
<td>11.3*</td>
<td>105</td>
</tr>
<tr>
<td>RYI</td>
<td>100</td>
<td>2448</td>
<td>24.5*</td>
<td>133</td>
</tr>
<tr>
<td>BL</td>
<td>180</td>
<td>36121</td>
<td>201*</td>
<td>253</td>
</tr>
<tr>
<td>MC</td>
<td>283</td>
<td>12238</td>
<td>43.2*</td>
<td>223</td>
</tr>
<tr>
<td>PH</td>
<td>268</td>
<td>11051</td>
<td>41.2*</td>
<td>198</td>
</tr>
<tr>
<td>GR</td>
<td>174</td>
<td>2285</td>
<td>13.2*</td>
<td>176</td>
</tr>
<tr>
<td>HA</td>
<td>206</td>
<td>6512</td>
<td>30.6*</td>
<td>142</td>
</tr>
<tr>
<td>MOR</td>
<td>160</td>
<td>1153</td>
<td>7.21*</td>
<td>142</td>
</tr>
</tbody>
</table>

* Lymphocyte transformation was determined to be present when the ratio of uptake of tritiated thymidine into DNA in the experimental cultures as compared with that of the negative cultures was greater than 3.0.
† The lymphocyte cultures included 0.1 ml. of the gastric mucosal homogenate preparation with an I.F. binding capacity of 30 ng. vitamin B$_{12}$ per ml.
‡ The lymphocyte cultures included 0.1 ml. of the gastric juice preparation with an I.F. binding capacity of 60 ng. vitamin B$_{12}$ per ml.
phocyte transformation did not occur in any of the cultures containing gastric juice and lymphocytes from the 18 control subjects.

Lymphocytes from one of 16 patients with pernicious anemia demonstrated lymphocyte transformation when cultured with 0.1 ml. of the gastric mucosal homogenate preparation (I.F. vitamin B₁₂ binding capacity of 30 ng./ml.). Lymphocytes from this patient also underwent transformation in the presence of the gastric juice and purified intrinsic factor preparations (above). Transformation was observed by lymphocytes from one of 18 control subjects whose lymphocytes were cultured with the gastric mucosal homogenate preparation.

Series B. Following long-term lymphocyte cultures (7 days) in the presence of 0.2 ml. of concentrated gastric juice (I.F. binding capacity 180 ng. vitamin B₁₂ per ml.) lymphocytes from 6 of 16 patients with pernicious anemia demonstrated lymphocyte transformation (Table 2). These 6 patients whose lymphocytes underwent transformation included the 3 patients whose lymphocytes were previously noted to transform in the presence of the purified intrinsic factor preparation. Under these conditions lymphocyte transformation was not observed in any of the cultures from the control subjects.

### Table 2.—Transformation of Lymphocytes* From Patients With Pernicious Anemia Cultured in the Presence of Concentrated Human Gastric Juice†

<table>
<thead>
<tr>
<th>Patient</th>
<th>cpm Negative Control</th>
<th>cpm PHA 1:10</th>
<th>cpm PHA 1:10</th>
<th>cpm PHA 1:10</th>
<th>cpm PHA 1:10</th>
<th>cpm With Concentrated Gastric Juice</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAR</td>
<td>111</td>
<td>36977</td>
<td>333*</td>
<td>580</td>
<td>5.26*</td>
<td></td>
</tr>
<tr>
<td>RE</td>
<td>418</td>
<td>13872</td>
<td>35.6*</td>
<td>2140</td>
<td>5.12*</td>
<td></td>
</tr>
<tr>
<td>KI</td>
<td>1201</td>
<td>16131</td>
<td>13.4*</td>
<td>18737</td>
<td>15.6*</td>
<td></td>
</tr>
<tr>
<td>CO</td>
<td>225</td>
<td>5508</td>
<td>25.8*</td>
<td>2199</td>
<td>9.77*</td>
<td></td>
</tr>
<tr>
<td>EV</td>
<td>525</td>
<td>15558</td>
<td>29.6*</td>
<td>1628</td>
<td>3.10*</td>
<td></td>
</tr>
<tr>
<td>OT</td>
<td>511</td>
<td>12553</td>
<td>24.6*</td>
<td>2140</td>
<td>4.19*</td>
<td></td>
</tr>
</tbody>
</table>

* Lymphocyte transformation was determined to be present when the ratio of uptake of tritiated thymidine into DNA in the experimental culture as compared with that of the negative control cultures was greater than 3.0.
† Concentrated human gastric juice, 0.2 ml. (I.F. binding capacity 180 ng. vitamin B₁₂ per ml.) was included in each lymphocyte culture.

Relationship of Humoral Autoantibodies to Lymphocyte Transformation.

There was no direct or inverse relationship between the detection of antiparietal cell antibodies and transformation of lymphocytes from patients with pernicious anemia. On the other hand there appeared to be an inverse relationship between lymphocyte transformation and the presence of circulating antibodies to intrinsic factor in the serum of the patients with pernicious anemia. Blocking antibodies to intrinsic factor were not demonstrable in the sera of any of the pernicious anemia patients whose lymphocytes exhibited transformation when cultured with the various gastric antigen preparations. Binding antibodies to intrinsic factor were detected in the serum of one patient with pernicious anemia whose lymphocytes exhibited transformation and
then only following prolonged incubation with the concentrated gastric juice preparation (Series B, above).

**Discussion**

Some of the best studied immunologic diseases in experimental animals result not from tissue damage effected by circulating antibodies but are secondary to lymphocyte-mediated sensitization.\(^{12}\) Cell mediated sensitization, i.e., of the delayed or tuberculin type, has not been previously reported in patients with pernicious anemia. The technics of lymphocyte transformation\(^{11-17}\) and macrophage immobilization\(^{20}\) have been proposed to serve as in vitro methods for detection of sensitization of lymphocytes. The value of these methods, if they genuinely reflect cell mediated sensitization, as in vitro methods to study lymphocytes from patients with diseases suspected to be autoimmune in nature is of obvious importance. We sought to see if lymphocytes of patients with pernicious anemia underwent transformation when exposed to antigens suspected to be important in the development of pernicious anemia.

The results of this study indicate that peripheral lymphocytes from some patients with pernicious anemia did show evidence of delayed hypersensitivity as judged by lymphocyte transformation. Lymphocyte transformation to human gastric juice and intrinsic factor preparations was exhibited only by those patients whose sera did not contain circulating blocking antibodies to intrinsic factor. In addition, of the six patients with pernicious anemia whose lymphocytes underwent transformation with the various gastric intrinsic factor-containing preparations serum from only one patient contained binding antibodies to intrinsic factor. On the other hand it was not possible to establish a direct or inverse relationship between lymphocyte transformation and the presence of antibodies to the gastric parietal cell antigen in the sera of the patients with pernicious anemia. A satisfactory explanation for the inverse relationship between lymphocyte transformation and intrinsic factor antibodies cannot be offered. It is possible that if I.F. is the antigen responsible for evoking lymphocyte transformation that humoral antibodies to intrinsic factor could inhibit the expression of lymphocyte transformation by competing for IF molecules with receptor sites on the surfaces of lymphocytes. However, the methods of purification and preparation of lymphocytes used in these studies would be anticipated to remove serum antibodies as well as usual varieties of cytophilic antibodies.\(^{24}\) A possibility which requires consideration, however, is that synthesis of antibodies to intrinsic factor may have occurred in vitro. Alternatively there may be fundamental differences in the nature and development of the immune responses expressed by those patients with either lymphocyte transformation or humoral antibodies to intrinsic factor.

The precise nature of the antigen, or antigens, responsible for eliciting transformation responses by the lymphocytes of certain of these patients with pernicious anemia cannot be deduced from these experiments. It is interesting to note that the proportion of patients with pernicious anemia whose lymphocytes exhibited transformation in the presence of the various gastric antigen preparations paralleled the intrinsic factor content of the lymphocyte cultures.
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Caution must be expressed in concluding that these data indicate that IF is the antigen responsible for evoking lymphocyte transformation observed in these patients with pernicious anemia. It is conceivable that other antigen(s) could have been purified in parallel with IF which although it was purified >200 fold in respect to protein concentration is not a completely chemically pure preparation.

These experimental data, indicating transformation of lymphocytes from certain patients with pernicious anemia cultures in the presence of a variety of gastric antigens, permit the consideration that delayed hypersensitivity mechanisms may be operative in pernicious anemia.

Table 3.—Incidence of Lymphocyte Transformation of Pernicious Anemia Patients With Various Gastric Antigen Preparations

<table>
<thead>
<tr>
<th>Antigen Preparation</th>
<th>I.F. Binding Capacity of Vitamin B12 Included in Each Lymphocyte Culture</th>
<th>% of Pernicious Anemia Patients Whose Lymphocytes Underwent Transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric mucosal homogenate</td>
<td>3 ng.</td>
<td>3.4 (1/29)</td>
</tr>
<tr>
<td>Gastric juice</td>
<td>6 ng.</td>
<td>6.9 (2/29)</td>
</tr>
<tr>
<td>Purified intrinsic factor</td>
<td>30 ng.</td>
<td>21.4 (3/14)</td>
</tr>
<tr>
<td>Concentrated gastric juice (long-term cultures)</td>
<td>36 ng.</td>
<td>37.5 (6/16)</td>
</tr>
</tbody>
</table>

SUMMARY

Experiments were directed to the investigation of evidence of a possible role of cellular immunity in patients with pernicious anemia. Lymphocyte-rich peripheral leucocyte preparations from 29 patients with pernicious anemia were cultured in the presence of a variety of preparations containing potential antigens: these included human gastric juice, human intrinsic factor (IF) preparations, and human gastric mucosal homogenates. Lymphocyte transformation was determined by measurement of the uptake of tritiated thymidine into DNA. Lymphocyte transformation occurred when lymphocytes from a portion of the patients with pernicious anemia (3.4 per cent to 37.5 per cent) were cultured in the presence of these antigen preparations. Lymphocyte transformation was noted in none of the patients whose sera contained blocking antibodies to intrinsic factor. There was no correlation between the presence or absence of lymphocyte transformation and the presence or absence of serum antibodies to the gastric parietal cell cytoplasmic antigen. These data on lymphocyte transformation permit the consideration that cellular immunity may participate in the pathogenesis of pernicious anemia.

SUMMARIO IN INTERLINGUA

Experimentos esseva effectuate pro investigar le evidencia de un rolo possibile de imunità cellular in patientes con anemia pernicioso. Preparationes de leucocytos peripheric ric in lymphocytos ab 29 patientes con anemia pernicioso esseva culturate in le presentia de un varietate de preparaciones continentie antijenos potential. Iste includeva human succo gastric, preparaciones de human factor intrinsec, e homogenatos de human mucosa gastric. Le transformation lymphocytic esseva determinate per mesurar le acception de thymidina a tritium ad in le acido deoxyribonucleari. Le transformation lymphocytic
occurreva quando lymphocytos ab un portion del patientes con anemia perniciose (3,4 pro cento a 37,5 pro cento) esseva culturate in le presentia de iste preparationes de antigeno. Le transformation lymphocytic esseva notate in nulle del patientes qui habeva in br seros anticorpore bloccante factor intrmnsec. Esseva constatate nulle correlation inter le presentia o absentia de transformation lymphocytic e le presentia o absentia de anticorpore seral anti le antigeno cytoplasmic de cellular parietal gastric. Iste datos relative al transformation lymphocytic permitte le conclusion que immunitate cellular participa possibilemente in le pathogenese de anemia perniciose.

REFERENCES

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