Production of TCII (Vitamin B₁₂ Transport Protein) by Mouse Mononuclear Phagocytes

By Bracha Rachmilewitz, Moshe Rachmilewitz, Malka Chaouat, and Michael Schlesinger

The role of the mononuclear-macrophage system in the production of transcobalamin II (TCII), the vitamin B₁₂ transport protein that delivers the vitamin to the tissues, was investigated in mice. Of all the organs examined for TCII content in unstimulated mice, highest TCII levels were found in the bone marrow. A considerable amount of TCII was present in peripheral blood monocytes. Small amounts of TCII were present in peritoneal exudate cells (PEC) and in the spleen. TCII was undetectable in the thymus and in lymph nodes. Following a single intraperitoneal injection of thioglycolate (TG), a significant increase of TCII concentration was observed in PEC, with a concomitant drop of TCII in the bone marrow. Cultures of PEC harvested from unstimulated and TG-stimulated mice synthesized and secreted considerable amounts of TCII into the medium. After a lag period of several hours the concentration of TCII in the culture medium increased constantly throughout the entire period of incubation (5–7 days). PEC from stimulated and unstimulated mice were separated into adherent and nonadherent populations. Only the adherent cells, i.e., the macrophages, were consistently found to produce TCII in vitro. These findings show that macrophages produce and secrete TCII. Macrophages, however, did not contain R binders (TCI and TCIII). The observation that concomitant with the rise of TCII in PEC following stimulation there was a marked fall of TCII in the bone marrow indicates that TCII is produced by precursors of mononuclear-macrophage cells in the bone marrow that migrate to the periphery.

Of the three vitamin B₁₂-binding proteins, the transcobalamin (TC) I, II and III, TCII has been shown to transport vitamin B₁₂ and to deliver it to the tissues.¹² The site of TCII synthesis is not yet established. Indirect evidence from animal experiments indicates that the liver and possibly other organs may be involved in the synthesis of TCII.³⁵ The transport function of TCII has been shown in vitro on cell systems such as Ehrlich ascites cells, reticulocytes, HeLa cells, transformed lymphocytes, fibroblasts, and mouse leukemic lymphoblasts.⁶⁻⁹ In man the role of TCII in delivering vitamin B₁₂ to tissues is evidenced by the observation that hereditary TCII deficiency leads to lack of cellular maturation of the hemopoietic system and neonatal megaloblastic anemia in spite of normal serum concentration of vitamin B₁₂.¹⁰⁻¹²

Increased serum TCII levels have been reported in acute leukemia and lymphoma in the active stage of the disease¹³,¹⁴ as well as in Gaucher disease.¹⁵,¹⁶

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Since increased serum TCII levels were observed in clinical conditions in which the common denominator was the stimulation of the reticuloendothelial system, which is now termed the mononuclear phagocyte system, it was suggested that the cells of this system (monocytes, macrophages) might be the site of TCII synthesis. This hypothesis was tested in an experimental model, the peritoneal exudate cells (PEC) of mice. Preliminary findings of this study were reported.

MATERIALS AND METHODS

Animals. Male inbred mice of the BALB/c strain, kept at the animal colony of the Department of Experimental Medicine and Cancer Research, were used.

Preparation of cell suspensions. PEC were obtained either from normal unstimulated mice or from mice injected with a single intraperitoneal (i.p.) injection of 2.5 ml 2.98%, thioglycolate. The cells were collected from the peritoneal cavity by repeated washings with saline. Bone marrow cells were obtained either from normal donors or from mice stimulated by a single i.p. injection of thioglycolate. To collect bone marrow cells the femurs of donor mice were dissected out and cut at both ends and the contents of the bone marrow cavity flushed out with saline by a syringe. Thioglycolate-stimulated mice used as a source for PEC and for bone marrow cells were killed at various time intervals after stimulation in sufficient numbers to provide the number of cells required for the respective experiment. Each experiment was repeated at least four times, so that each point in the figures of experiments both in vivo and in vitro represents an average of at least four results. The types of cells collected from mouse peritoneal cavity were similar to those reported by Gordon et al. In the unstimulated mice the PEC contained about 40%, macrophages and 60%, lymphocytes; the PEC of thioglycolate-stimulated mice contained over 70%, macrophages and less than 30%, lymphocytes. Suspensions of cells from various lymphoid organs (thymus, spleen, lymph nodes) were prepared as described previously.

Mouse peripheral blood cells were separated by a modification of the Böyum method. Heparinized blood, collected from the paraorbital vein of mice, was layered on top of a barrier consisting of 4 ml 9%, Ficoll (Pharmacia) dissolved in Hypaque (Wintrobe, N.Y.), diluted 1:4 in distilled water. The test tubes were centrifuged at 1400 rpm for 20 min. Cells remaining at the interface on top of the barrier (lymphocyte-monocyte fraction) and at the bottom of the test tube (granulocyte fraction) were collected carefully.

Preparation of cell sonicates. All cell suspensions were exposed to 0.83%, NH₄Cl according to the method of Boyle to lyse red blood cells. Finally, the cells were suspended in a volume of 2 ml saline. The number of cells was determined in each sample, and the cells were disintegrated by ultrasonic vibration for 3 min. The sonicates were centrifuged for 10 min at 12,000 rpm, and the supernates were separated.

Organ homogenates. Homogenates of brain, liver, and kidney were exposed to 0.83%, NH₄Cl, washed, and spun at 3000 rpm for 10 min, and the sediments were collected. Saline (2 ml) was added to the sediments, which were then resuspended and exposed to ultrasonic vibration as described above. The sonicates were spun at 3000 rpm for 10 min, and the supernatant fluids were harvested. The optical density of the supernates was determined at 280 nm, and the TCII content was assayed in each sample. In some experiments similar homogenates were prepared from cells of the peritoneal cavity, spleen, and bone marrow for determination of TCII content.

Cell cultures. PEC from either normal or stimulated mice were suspended in MEM-S culture medium (Grand Island Biological) supplemented with 10%, fetal calf serum (Grand Island), penicillin (100 U/ml), and streptomycin (100 μg/ml). In a few experiments Hank’s culture medium (Bio-Lab, Jerusalem) was used instead of MEM-S. The use of culture media supplemented with vitamin B₁₂ had to be avoided to prevent interference with the assay of TCII. Cell suspensions (2 ml) containing 2 × 10⁶ cells/ml were placed in 30-mm plastic Petri dishes (Falcon) and incubated in a humidified atmosphere containing 5% CO₂ in air at 37°C. The supernatant fluid was collected at various time intervals after the cultures were set up and was assayed for TCII content.
The cells grown in culture consisted of either the total population of PEC or the adherent and nonadherent subpopulations. Separation into these subpopulations was achieved by letting the PEC attach to plastic petri dishes for 3 hr at 37°C. The nonadherent cells were then collected with a Pasteur pipette and kept in culture separately. The adherent cell population derived from TG-stimulated mice as well as from unstimulated mice contained over 95% macrophages as identified by examination of Giemsa-stained dishes. Fresh culture medium was added to the cells adhering to the plastic dishes and to the nonadherent cells, and both cell types were incubated for varying time intervals. At the end of the incubation period the supernatant fluid was separated and the content of TCII was determined.

Assay of TCII. For the determination of TCII in cell sonicates a volume of sonicate containing the equivalent of 2 x 10⁶ cells was used. The TCII content of tissue sonicates was assayed in volumes varying from 25 to 100 μl. The samples were labeled with 57CoB₁₂, using a solution containing 10,000 pg/mI 57CoB₁₂ (Radiochemical Centre, Amersham; specific activity 100-130 Ci/g). The minimum amount of radioactive vitamin B₁₂ added was 100 pg 57CoB₁₂. Higher amounts of radioactive vitamin B₁₂ were added whenever required to provide full saturation of the B₁₂ binders. After incubation for 10 mm at 37°C in a shaking bath, which allowed the 57CoB₁₂ binding, the B₁₂ protein binders were separated quantitatively by using the charged cellulose filter technique described by Selhub et al. The principle of the technique is as follows: The 57CoB₁₂-labeled solution is passed through a stack of charged cellulose filters composed of one negatively charged cellulose nitrate filter (Shleicher & Schull; porosity 0.45, 25 mm diam) and three DEAE filters (Whatman DE-81; 25 mm diam). The DEAE filters used were chemically identical with the granular Whatman DE-52 used for chromatographic separation of the transcobalamins. TCII adsorbs selectively and quantitatively to the cellulose nitrate filter. TCΙ and TCΙΙΙ, being negatively charged molecules, pass through the negatively charged cellulose nitrate filter and bind chemically to the DE-81 filters. The lower retention of TCΙΙΙ by the DE-81 filters allows specific desorption of TCΙΙΙ from a duplicate stack of filters with a low ionic strength solution. Excessive CoB₁₂ was removed by repeated washings of the filters with the buffer solution used in this system, borate buffer 0.1 M pH 8.5. The filters were separated after the washings and their radioactivity was determined for 1 mm in a well-type scintillation counter. The results were expressed either in cpm of the radioactivity retained on the filters or as ng vitamin B₁₂ bound/2 x 10⁶ cells. Since the molar ratio binding between the B₁₂ molecule and the transcobalamin molecules is 1:1, the amount of TCII was expressed as ng vitamin B₁₂ bound.

Mouse serum contains very high concentrations of TCII that might obscure the detection of the minute amounts of the R binders (TCΙ and TCΙΙΙ). It was therefore necessary to dilute the mouse serum 100-fold in saline before determination of the B₁₂ binders.

Procedures Used to Determine Similarity Between Mouse and Human TCII

The identification of serum and PEC mouse TCII and its similarity to human serum TCII were established in our laboratory according to the following accepted criteria:

1/ Gel filtration on Sephadex G-200 columns. Using the procedure of Hom and Olesen, human TCII, mouse serum, and PEC TCII showed identical elution patterns and similar molecular weights of about 38,000 daltons.

2/ DEAE-cellulose chromatography columns. Similar to human TCII, mouse TCII of both serum and PEC was eluted with phosphate buffer 0.06 M when the stepwise elution method was used. TCΙ of both human and mouse sera was eluted with phosphate buffer 0.15 M, while in PEC extracts only TCII was detected.

3) QuSo-silica gel adsorption and ammonium sulfate precipitation. Mouse serum and PEC TCII were found to adsorb to QuSo-G32-silica gel, with use of the technique of Jacob et al., and to precipitate specifically like human TCII with ammonium sulfate, with Carmel’s technique. Mouse and human serum from which TCII was adsorbed by QuSo-G32 contained only the R binders, detected on the DE-81 filters.

4) Unsaturated B₁₂ binding capacity (UBBC) determination. UBBC determination of both human and mouse sera and PEC, using different techniques, showed that mouse TCII, like human TCII, carry the majority of the unsaturated B₁₂ binding capacity, which determines the UBBC of the serum.
Table 1. Concentration of TCII in Sonicates of Various Cells and Organ Homogenates of Unstimulated Mice

<table>
<thead>
<tr>
<th>Cells and Organs Tested</th>
<th>Concentration of TCII</th>
<th>Concentration of R Binders</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pg/I x 10^6 Cells</td>
<td>pg/mg Protein</td>
</tr>
<tr>
<td>Bone marrow cells</td>
<td>32.3</td>
<td>459.6</td>
</tr>
<tr>
<td>Peripheral blood</td>
<td>6.7</td>
<td>Not done</td>
</tr>
<tr>
<td>monocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peritoneal exudate cells</td>
<td>1.8</td>
<td>11.1</td>
</tr>
<tr>
<td>Spleen cells</td>
<td>1.0</td>
<td>32.3</td>
</tr>
<tr>
<td>Peripheral blood granulocytes</td>
<td>0</td>
<td>23.3</td>
</tr>
<tr>
<td>Lymph node cells</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Thymus cells</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Liver</td>
<td>0-2.1</td>
<td>0</td>
</tr>
<tr>
<td>Kidney</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Brain</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Serum*</td>
<td>510-860</td>
<td>21.6-26.6 pg/mg protein</td>
</tr>
</tbody>
</table>

*Serum TCII concentration per ml: 31,000-52,000 pg; serum R binders concentration per ml: 1300-1600 pg.

(5) TCII identification in eluates from cellulose nitrate filters. Eluates obtained from the negatively charged cellulose nitrate filters, through which human or mouse sera were passed, were tested for their UBBC and for their behavior on DEAE chromatography columns and Sephadex G-200 columns. Mouse TCII eluates, like human TCII eluates, were found to carry the majority of the unsaturated B12-binding sites and to have characteristic TCII elution patterns on both DEAE and Sephadex G-200 columns.

Additional indication for the similarity of mouse and human TCII was obtained by Tan and Hansen using zirconyl phosphate gel binding and by Tan and Blaisdell, who showed biologic and immunologic cross reactivity between mouse and human TCII. Mouse serum fractions eluted from the DE-81 filters had characteristic R binder fractionation patterns on both DEAE and Sephadex G-200 columns.

RESULTS

Concentration of TCII in various organs in unstimulated mice. Sonicates of cell suspensions were prepared from lymphoid organs and assayed for TCII levels. Low concentrations of TCII were detected in sonicates of peritoneal exudate cells (PEC) and spleen cells from unstimulated animals (Table 1). The concentration of TCII/mg protein was higher in spleen cells than in PEC. In contrast, the concentration of TCII per cell was somewhat higher in PEC than in spleen cells, probably reflecting the larger cell size of PEC. No TCII was detected in cells of the mesenteric and peripheral lymph nodes or in the thymus. In homogenates of the kidney and brain TCII was undetectable. Liver homogenates contained low, irregular levels of TCII. High concentrations of TCII were found in bone marrow cells.

A fraction of peripheral blood, enriched for lymphocytes and monocytes, was also found to contain considerable amounts of TCII but no R binders (Table 1). The reverse was true for the granulocyte fraction separated from peripheral blood. The latter fraction contained considerable quantities of TC1 and TCIII but very little TCII. The concentrations of TCII and R binders in mouse serum as determined on charged cellulose filters and compared with
DEAE chromatography and Sephadex G-200 filtration are summarized in Table 1.

**Effect of thioglycolate stimulation on TCII levels in PEC.** The concentration of TCII in PEC was considerably increased 4 hr after a single i.p. injection of thioglycolate (Fig. 1). The level of TCII continued to increase 8 hr after stimulation and reached a peak at 24 hr. At that point TCII reached a concentration about 100-fold higher than the initial TCII concentration in the unstimulated PEC. A precipitous fall of the TCII level in PEC was observed after 48 hr. Thereafter TCII concentration in PEC remained similar to that in unstimulated cells.

The changes in the number of PEC after stimulation did not correlate with the changes in the TCII content. While the concentration of TCII dropped to almost normal levels 48 hr after stimulation, the number of PEC continued to increase, reaching a peak on day 4 (Fig. 1).

**Effect of thioglycolate stimulation on TCII in bone marrow cells.** Parallel to TCII determination in PEC, the level of TCII was determined in bone marrow cells at various time intervals after thioglycolate stimulation. Prior to stimulation, bone marrow cells contained high levels of TCII. Within 4 hr after stimulation a slight drop in the concentration of TCII was detected in the bone marrow (Fig. 2). At 8 hr after stimulation the TCII level dropped markedly. At 24 hr the level of TCII in the bone marrow cells was almost completely recovered and continued to increase during the following 24 hr. No significant changes were found in the number of the bone marrow cells during this period.

**TCII production in vitro.** PEC from either normal or stimulated mice were cultured in vitro, and TCII levels were determined in cell sonicates and in the medium. TCII concentration in the cells remained constantly low all through the incubation period regardless of the source of the cells (less than 0.01 ng TCII/2 x 10^6 cells). In contrast to the constant low intracellular levels of TCII, extracellular TCII increased daily. TCII became detectable in the culture medium after a lag period of > 4 hr (Fig. 3). The production and release of
TCII into the medium was similar in unstimulated PEC and in PEC harvested 24 hr after thioglycolate stimulation (Fig. 4). PEC harvested 4 days after thioglycolate stimulation secreted even larger quantities of TCII (Fig. 6) in spite of the fact that in vivo at this stage the TCII content of the cells decreased to that of unstimulated cells (Fig. 1).

Nature of TCII-producing cells. In order to identify which cell subpopulation produces TCII, PEC were separated according to their capacity to adhere to a plastic surface. Adherent cells isolated from the peritoneal cavity of either unstimulated mice or mice stimulated in vivo for 24 hr produced similar, high quantities of TCII throughout the incubation period (Fig. 5). Even larger
quantities of TCII were produced and secreted by adherent PEC separated from mice 4 days after stimulation (Fig. 6). The amount of TCII produced by the adherent cells was, in all experiments, almost as high as that produced by the total unseparated population of PEC.

Nonadherent cells, from either normal mice or from animals stimulated for 24 hr failed to produce significant amounts of TCII (Fig. 5). The nonadherent cells separated from PEC harvested 4 days after thioglycolate stimulation produced small amounts of TCII, considerably lower than the adherent cells.

DISCUSSION

Mouse and human mononuclear cells have been shown to synthesize and secrete a number of biologically active substances. These include pyrogens, lysosomal hydrolases, lysozyme, complement factors, plasminogen activators, and prostaglandins. It has been suggested that cells of the human reticuloendothelial system might be involved in the metabolism of ferritin and haptoglobin. The present study stemmed from clinical observations that suggested that cells of the mononuclear phagocyte system might be the site of TCII production.
The experiments performed both in vivo and in vitro using mouse PEC show conclusively that mouse TCII, which has been shown to be similar to human TCII, is produced and released by mouse peritoneal macrophages. The experiments in vivo showed that while only small amounts of TCII were present in PEC of unstimulated mice, the TCII concentration increased up to about 100-fold 24 hr after stimulation with a single injection of thioglycolate. Synthesis and secretion of TCII by mouse PEC was shown also in the experiments in vitro. The production and release of TCII was shown to be limited to the adherent cells only, i.e., macrophages. The nonadherent cell population did not contain TCII. Mouse peripheral blood granulocytes did not contain TCII but were found to contain only R binders.

The kinetics of TCII production and secretion by mouse PEC in vitro was found to resemble the production and secretion of lysozyme by mouse PEC and human monocytes as described by Gordon et al. As in lysozyme production, there was an initial lag of a few hours in the secretion of TCII. Thereafter the rate of secretion of the two substances remained constant under optimal culture conditions. Another point of similarity was that while the concentration of these substances in the medium increased constantly, the intracellular content remained low throughout the incubation period.

In contrast to the continuous production of TCII in vitro, PEC in vivo contained an increased level of TCII for only 24 hr after thioglycolate stimulation. This apparent difference could be explained in a number of ways. One possibility is that PEC cultured in vitro are exposed to continuous stimulation either by substances present in the medium or by contact with the plastic surface. This contention is supported by the observation (unpublished data) that a second thioglycolate injection, given when the TCII level declines, was again followed by an increase of TCII in PEC in vivo. An alternative explanation is...
that the drop of TCII in PEC harvested 24 hr after stimulation may result not from a decreased production of TCII but rather from an increased capacity to secrete the substance.

Of all organs examined in the unstimulated animal, the bone marrow was found to contain the highest concentration of TCII. Peripheral blood monocytes also contained a considerable amount of TCII, less than bone marrow cells but more than PEC (Table 1). It is well established that the progenitors of the mononuclear phagocyte system are situated in the bone marrow and give rise to blood monocytes, PEC, and Kupfer cells as well as other tissue macrophages that migrate from the bone marrow via the blood to the respective tissues and accumulate at sites of inflammatory and immunologic reactions. Our findings that mouse peritoneal macrophages produce TCII and the fact that macrophages originate from bone marrow lend support to the conclusion that the high levels of TCII found in the bone marrow cells are most probably produced by the progenitors of the peritoneal macrophages. The observation that concomitant with the rise of TCII concentration in the peritoneal macrophages following stimulation, there was a marked fall of TCII in the bone marrow cells provides further support to our conclusion that the TCII-producing cells in the peritoneum originate from the bone marrow. TCII may be used as a biochemical marker for tracing the route of mononuclear cells from the bone marrow.

Tan and Hansen suggested the liver as the site of TCII production. This conclusion was based on experiments performed on perfused mouse liver. Since the liver consists of a heterogenous cell population, it is possible that the Kupfer cells in the liver were the cells that produced TCII.

The demonstration in the present study that mouse macrophages produce and secrete TCII supports our assumption that the high TCII levels found in diseases associated with stimulation and proliferation of the mononuclear phagocyte system could well be a result of increased TCII production by cells of this system.

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