Retinoic Acid-induced Expression of Tissue Transglutaminase in Mouse Peritoneal Macrophages*

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The culture of peritoneal macrophages in serum-containing media induces a dramatic increase in the expression of the enzyme tissue transglutaminase. The transglutaminase-inducing activity of serum isabolished by extraction of lipids and fully restored by re-addition of physiological concentrations (1–100 nm) of trans-retinoic acid. Induction of the enzyme is detectable within a 90-min exposure of macrophages to retinoic acid and is completely blocked by actinomycin D, suggesting that the retinoid rapidly increases the rate of transglutaminase gene expression. Delipidized serum is required to elicit the transglutaminase-inducing activity of retinoic acid and this effect is decreased if the serum is depleted of the serum retinol-binding protein. Our studies suggest that retinoic acid and serum retinol-binding protein can directly regulate macrophage gene expression and specifically induce the synthesis of tissue transglutaminase.

Transglutaminases are a group of enzymes that covalently cross-link proteins by catalyzing the formation of ε-(γ-glutamyl)lysine isopeptide bonds between protein-bound lysine and glutamine residues (for a review see Folk and Finlayson, 1977; Folk, 1980). Although different transglutaminases appear to be very similar in their substrate specificity, several distinct forms of the enzyme have been identified (Folk, 1980). Some transglutaminases such as plasma factor XIII or the transglutaminase of rodent seminal plasma are extracellular enzymes whereas other transglutaminases are found exclusively inside cells. Tissue transglutaminase is an intracellular enzyme that is found in a variety of cultured cells and tissues (Connellan et al., 1971; Birckbichler and Patterson, 1978; Murtaugh et al., 1983). The enzyme is an 80,000-Da polypeptide that is responsible for the formation of the isopeptide bonds found in normal cultured cells (Birckbichler and Patterson, 1978). We have been interested in the role that tissue transglutaminase may play in the function of immune cells, particularly macrophages and monocytes. Several laboratories have reported that the activation of macrophages in vivo is associated with large increases in transglutaminase activity (Fesus et al., 1981; Schroff et al., 1981; Leu et al., 1982; Murtaugh et al., 1983) suggesting that this increase in enzyme activity contributes to the enhanced functional capacity of activated macrophages (Fesus, 1982).

To investigate the regulation of tissue transglutaminase activity in macrophages and monocytes, we have studied the expression of the enzyme in cells cultured in vitro (Murtaugh et al., 1983; Mehta et al., 1984; Murtaugh et al., 1984). Both monocytes and macrophages accumulated large amounts of tissue transglutaminase when cultured in the presence of serum-containing media. In mouse resident peritoneal macrophages, an increased level of transglutaminase activity could be detected within 90 min of exposing the cells to serum-containing media. The induction continued for at least 24 h, and the levels of the enzyme increased 150-fold until it accounted for 1–2% of total cellular protein (Murtaugh et al., 1983). This very large induction was also remarkably specific. Tissue transglutaminase was the only major protein altered by the culture of the cells in serum-containing medium (Murtaugh et al., 1983). The isolation and identification of the factor in mouse serum responsible for the induction of tissue transglutaminase should provide a useful insight into the mechanisms involved in the regulation of this enzyme and macrophage gene expression in general. The studies we report here suggest that two components of mouse serum, trans-retinoic acid and SRBP,† account for the ability of serum to regulate the rate of transglutaminase expression in macrophages.

EXPERIMENTAL PROCEDURES

Materials—Adult ICR strain male mice were obtained from Tumco (Houston, TX). Rabbit anti-human retinol-binding protein immunoglobulin was purchased from Boehringer Mannheim (Indianapolis, IN). Goat anti-tissue transglutaminase antibodies were prepared by immunization of a goat with purified guinea pig liver tissue transglutaminase. The anti-transglutaminase antibodies were purified by affinity chromatography on a column of rat liver tissue transglutaminase immobilized on CNBr-activated Sepharose. The purified antibodies were directly iodinated with chloramine-T and NaI[125]I. The conditions for preparation, purification, and characterization of these antibodies have been described in detail elsewhere (Murtaugh et al., 1983).

trans-Retinol and trans-retinoic acid were purchased from Sigma (St. Louis, MO). We have also used chromatographically pure trans-retinoic acid obtained through the auspices of Dr. Michael Sherman (Hoffmann-LaRoche Inc., Nutley, NJ) with comparable results. The cis-retinoic acid used was also generously provided by Hoffmann-LaRoche Inc. All solutions of retinoids were made up in subdued light, and stored in ethanol at ~20 °C in the dark. Bacterial lipopolysaccharide B, Escherichia coli 0111:B4 was purchased from Difco (Detroit, MI). 12-O-Tetradecanoylphorbol-13-acetate was purchased from Consolidated Midland Corp. (Brewer, NY). [3H]Putrescine and [35S]-protein A were purchased from New England Nuclear (Boston, MA). Na[125]I and [35S]methionine were purchased from American (Arlington Heights, IL), and [3H]labeled amino acids were from ICN (Irvine, CA).

† The abbreviations and trivial name used are: SRBP, serum retinol-binding protein; SDS, sodium dodecyl sulfate; cis-retinoic acid, 13-cis-retinoic acid.

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Preparation of Cells—Mouse peritoneal macrophages were prepared as described previously (Murtaugh et al., 1983). The peritoneal cavity was lavaged with chilled, heparinized saline and the mixture of peritoneal cells obtained was washed once by centrifugation and resuspension in RPMI medium. Peritoneal macrophages, purified on the basis of their rapid attachment to plastic tissue culture dishes, were prepared under the various experimental conditions in RPMI 1640 media in a 37 °C incubator in an atmosphere of 95% air and 5% CO₂.

To test the effects of phagocytosis on the induction of tissue transglutaminase, monolayers of cells were incubated for 60 min with a suspension of IgG-coated multilamellar vesicles in RPMI media (Melta et al., 1984). The cells were then washed three times with prewarmed RPMI and the subsequent induction of tissue transglutaminase was measured following incubation in serum or retinoid-containing media as described below.

Preparation and Storage of Sera—Fresh mouse blood was collected by cardiac puncture and allowed to clot in glass tubes. Serum from several animals was pooled and stored frozen at -20 °C for 30 days or less. Human blood was collected by venipuncture from a healthy volunteer. Serum was obtained following coagulation of whole blood and was also stored in aliquots at -20 °C and used within 30 days. Fetal calf serum was purchased from Sterile Systems (Los Angeles, CA) and mouse serum was purchased from Pel-Freez (Rogers, AR). The duration of storage of these commercially obtained sera is not known.

Delipidization of serum was carried out using the method of Rothblat et al. (1976). Intact serum (25 ml) was precipitated with 250 ml of acetone/ethanol (1:1). The precipitate was filtered on No. 1 Whatman filter paper, washed with 25 ml of diethyl ether, and air dried. The precipitated proteins were then dissolved in 25 ml of RPMI medium and insoluble debris was removed by centrifugation.

To remove retinol-binding protein from delipidized serum, 0.5 ml of delipidized mouse serum (50 mg of protein) was incubated for 20 h at 4 °C with 5 μl of rabbit anti-human SRBP immunoglobulin or normal rabbit IgG. Immune complexes were removed by low speed centrifugation after adsorption to heat-killed Staphylococcus aureus (Pansorbin, Calbiochem) introduced as a 2.5-ml suspension in phosphate-buffered normal saline.

Measurement of Tissue Transglutaminase in Macrophage Extracts—Transglutaminase activity was assayed by measuring the Ca²⁺-dependent incorporation of [³H]putrescine into casein (Lordan et al., 1972). Cell lysates, containing 0.5% Triton X-100, were incubated with N,N-dimethylcasein (2 mg/ml) and [³H]putrescine (0.5 mm) in a buffer containing 20 mM Tris-HCl, 5 mM CaCl₂, 15 mM β-mercaptoethanol and 150 mM NaCl. Aliquots were taken at intervals and spotted onto Whatman 3MM filter papers, and the protein-bound radioactivity was determined by trichloroacetic acid precipitation (Lordan et al., 1972). Enzyme activity was expressed as picomoles of [³H]putrescine incorporated into casein per min of mg of macrophage extract protein. Proteins were determined by the Coomassie blue assay (Bradford, 1976).

To measure the amount of tissue transglutaminase in cell extracts, monolayers of macrophages were solubilized directly in SDS-containing electrophoresis buffer and boiled. The extract was fractionated on a 6.5% discontinuous polyacrylamide slab gel using the method of Blatter et al. (1972). Proteins were then electroblotted onto nitrocellulose filters and the filters were saturated with a bovine serum albumin-containing solution (Murtaugh et al., 1963). Tissue transglutaminase was then detected by treating the filter with an iodinated antibody to tissue transglutaminase, followed by extensive washing and autoradiography.

Since tissue transglutaminase was the only immunoreactive protein in macrophage extracts, we also developed an immunoblot assay for the enzyme that bypassed the need for an electrophoretic fractionation of proteins prior to reaction with the anti-transglutaminase antibody. Macrophages (7.5 × 10⁵ cells/50 μl) were plated in individual wells of a flat-bottom 96-well tissue culture dish (96-well half-area tissue culture plates) at a density of 4×10⁴ cells/ml [⁵¹]methionine. The plates were tightly covered with a sheet of nitrocellulose backed by a sheet of polyethylene followed by sheets of blotting paper, this assembly being clamped between the tissue culture plate bottom and top. The lysate was transferred to the filter by inverting the plate and allowing an aliquot of the lysate to adsorb for 30 min. Excess liquid was allowed to drain from the nitrocellulose by reinverting the clamped assembly for an additional 30 min. The nitrocellulose was then saturated with a bovine serum albumin solution as described above, and the filter was treated with the iodinated anti-tissue transglutaminase antibody. The amount of tissue transglutaminase in the cells was estimated from the intensity of the autoradiographic spot corresponding to the site of tissue transglutaminase. Aliquots of purified guinea pig liver transglutaminase of known concentrations were added to unstained cell lysates as standards.

Immunoprecipitation of Metabolically Labeled Cell Extracts—Monolayers of macrophages were metabolically labeled either by culture for 20 h in media supplemented with 10 μCi/ml [³⁵S]methionine or by culture for 20 min in soytone-free media containing 100 μCi/ml [³⁵S]methionine. The monolayers were then washed and solubilized in a lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS. Tissue transglutaminase was precipitated by the addition of affinity purified anti-transglutaminase antibodies (50 μg) or an equivalent amount of pre-immune IgG. The sample was incubated at 4 °C for 18 h and then immune complexes were precipitated by addition of SDS-washed, heat-killed S. aureus. Precipitated proteins, solubilized in an SDS-containing electrophoresis buffer, were fractionated on a 6.5% discontinuous polyacrylamide slab gel and the radioiodolated proteins were identified by fluorography (Bonner and Laskey, 1974).

RESULTS

Role of Retinoic Acid in the Induction of Tissue Transglutaminase—Previous studies indicated that the transglutaminase-inducing activity of fresh mouse serum was heat labile and nondialyzable (Murtaugh et al., 1983). Our initial attempts to fractionate serum and identify the active component revealed that the activity was unpredictably unstable and was readily lost with purification. In order to gain some insight into the lability of the factor, we subjected serum to several manipulations designed to inactivate specific serum components. Unexpectedly, conditions such as mild heating (56 °C for 30 min) or denaturants such as urea that perturbed protein structure had little effect on the transglutaminase-inducing activity of serum. Precipitation with organic solvents, on the other hand, completely abolished the inducing activity. To examine the role of lipid-extractable molecules in the induction of tissue transglutaminase, we delipidized mouse serum by first precipitating the serum proteins with aceton and then washing the precipitated proteins with diethyl ether (Rothblat et al., 1976). Macrophages were then cultured for 20 h in serum-free media alone, fresh mouse serum, or serum that had been delipidized as described above. The transglutaminase activity in these cells is shown in Fig. 1A. Cells cultured for 20 h in serum-free media had a transglutaminase activity of 400 pmol/min/mg. This activity increased 15-fold when the cells were cultured in media that contained fresh mouse serum. If the serum was first delipidized, then all inducing activity was lost and the accumulation of the enzyme was no greater than in cells cultured in serum-free media alone (Fig. 1A).

The low amount of transglutaminase activity in macrophages cultured in delipidized serum was due to the failure of the delipidized serum to induce the expression of tissue transglutaminase. Freshly isolated mouse peritoneal macrophages were exposed to varying concentrations of intact or delipidized mouse serum and the levels of tissue transglutaminase were determined by an immunoblot assay of the cell lysate (Fig. 1B).
could restore the ability of delipidized mouse serum to induce expression of tissue transglutaminase. Addition of cholesterol, lecithin, testosterone, and stearic acid (each at 100 µM) to delipidized mouse serum had no effect on its transglutaminase activity, while the addition of retinoic acid (1 nM) completely restored the ability of delipidized mouse serum to induce expression of tissue transglutaminase in cell lysates determined by immunoblot analysis with an anti-transglutaminase antibody as described under "Experimental Procedures."

FIG. 2 (right). Retinoic acid-induced expression of tissue transglutaminase in different culture media. Macrophages were cultured for 20 h with different concentrations of retinoic acid (RA) either in RPMI plus 10% delipidized mouse serum (row 1), RPMI media alone (rows 2A and 2B), and RPMI plus 1% bovine serum albumin (rows 3A and 3B). The level of tissue transglutaminase was determined by immunoblot assay. Rows 1, 2A, and 3A are the autoradiograms of the immunoblots obtained after a 12-h exposure. Rows 2B and 3B are autoradiograms of the same immunoblots exposed for 96 h.

TABLE I

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Transglutaminase activity pmol/min/mg</th>
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<tbody>
<tr>
<td>RPMI 1640 alone</td>
<td>265 ± 43</td>
</tr>
<tr>
<td>RPMI 1640, 10% delipidized mouse serum</td>
<td>577 ± 85</td>
</tr>
<tr>
<td>RPMI 1640, 10 nM retinoic acid</td>
<td>1111 ± 85</td>
</tr>
<tr>
<td>RPMI 1640, 1% bovine serum albumin, 10 nM retinoic acid</td>
<td>1514 ± 100</td>
</tr>
<tr>
<td>RPMI 1640, 10% delipidized mouse serum, 10 nM retinoic acid</td>
<td>9271 ± 485</td>
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* Monolayers of macrophages were cultured for 20 h under the indicated conditions.

In heavily exposed autoradiograms (rows 2B and 3B), it was apparent that high concentrations of retinoic acid added to medium alone or medium plus serum albumin could induce a relatively small increase in the levels of the enzyme. A quantitative comparison of the transglutaminase-inducing activity of retinoic acid added to albumin-containing medium and delipidized mouse serum is shown in Table I. Cells cultured in RPMI 1640 medium alone or medium containing delipidized serum had very little transglutaminase activity (280–850 pmol/min/mg). Retinoic acid (10 nM) added either to serum-free medium or medium containing 1% serum albumin had little effect on the induction of tissue transglutaminase. The experiment shown in Fig. 2 compared the transglutaminase-inducing activity of different concentrations of retinoic acid added to delipidized serum (row 1), RPMI 1640 medium alone (rows 2A and 2B), or RPMI plus 1% serum albumin (rows 3A and 3B). Retinoic acid added to delipidized serum induced a concentration-dependent accumulation of tissue transglutaminase in the treated cells (row 1). The threshold of this effect was less than 1 nM and the induction was concentration-dependent. Retinoic acid added to either medium alone or medium plus albumin was much less effective in inducing expression of the enzyme (rows 2A and 3A). In heavily exposed autoradiograms (rows 2B and 3B), it was apparent that high concentrations of retinoic acid added to medium alone or medium plus serum albumin could induce a relatively small increase in the levels of the enzyme. A quantitative comparison of the transglutaminase-inducing activity of retinoic acid added to albumin-containing medium and delipidized mouse serum is shown in Table I. Cells cultured in RPMI 1640 medium alone or medium containing delipidized serum had very little transglutaminase activity (280–850 pmol/min/mg). Retinoic acid (10 nM) added either to serum-free medium or medium containing 1% serum albumin had little effect on the induction of tissue transglutaminase. The experiment shown in Fig. 2 compared the transglutaminase-inducing activity of different concentrations of retinoic acid added to delipidized serum (row 1), RPMI 1640 medium alone (rows 2A and 2B), or RPMI plus 1% serum albumin (rows 3A and 3B). Retinoic acid added to delipidized serum induced a concentration-dependent accumulation of tissue transglutaminase in the treated cells (row 1). The threshold of this effect was less than 1 nM and the induction was concentration-dependent. Retinoic acid added to either medium alone or medium plus albumin was much less effective in inducing expression of the enzyme (rows 2A and 3A).
bumin produced a small increase in the activity of the enzyme (1100–1500 pmol/min/mg). The same concentration of retinoic acid added to media containing 10% delipidized mouse serum induced a very large increase in the levels of the enzyme. In this particular experiment, the enzyme activity increased to 9300 pmol/min/mg.

To be certain that the immunoreactive material induced by retinoic acid was tissue transglutaminase, cells were cultured in delipidized serum alone or delipidized serum plus retinoic acid. The cells were then solubilized in an SDS-containing buffer, the extract was fractionated by electrophoresis on a 6.5% slab gel, and the mobility of the immunoreactive material was compared with purified tissue transglutaminase. As can be seen in Fig. 2B, retinoic acid induced expression of a single immunoreactive polypeptide that co-migrated with purified tissue transglutaminase.

Macrophages cultured in delipidized mouse serum responded to very low levels of retinoic acid (Fig. 3A). Concentrations as low as 0.1 nM produced a detectable increase in macrophage transglutaminase activity. Progressively larger accumulations were observed at concentrations up to 10 nM. Even though the cellular response to retinoic acid appeared to approach saturation at 10 nM, the dose-response curve was broad. As can be seen in Fig. 3B, increasing the concentration of trans-retinoic acid to 100 nM and even 1000 nM further increased the accumulation of transglutaminase. Concentrations of retinoic acid greater than 1 μM produced signs of cellular toxicity and could not be reliably evaluated.

The trans isomer of retinoic acid was more active than either the cis form or the alcohol derivative, retinol, in inducing macrophage transglutaminase activity. Fig. 3B compares the potency of cis- and trans-retinoic acid and retinol as inducers of macrophage transglutaminase. The dose-response curve for cis-retinoic acid paralleled the curve of the trans isomer but was shifted to the right. cis-Retinoic acid was only 3% as active as trans-retinoic acid in inducing expression of macrophage transglutaminase. Retinol was even less active. As can be seen in Fig. 3B, only 1 μM retinol induced significant accumulations of tissue transglutaminase, indicating this retinoid was only 0.1% as active as trans-retinoic acid.

To determine how rapidly retinoic acid induced the expression of tissue transglutaminase, the rate of enzyme synthesis was measured by immunoprecipitation of cells pulse-labeled with [35S]methionine. Fig. 4 shows the result of such an experiment. Lane 1 contains the immunoprecipitate obtained with pre-immune IgG. The only band seen is below the shadow of the heavy chain of IgG and we believe it is macrophage actin nonspecifically adsorbed by the immune complexes. Lane 2 shows the immunoprecipitate obtained from control cells cultured for 4 h in delipidized mouse serum alone. A faint band of tissue transglutaminase is detectable (arrow). Lanes 3, 4, and 5 contain the immunoprecipitates of cells incubated for 0.5, 1, and 2 h in the presence of retinoic acid. The intensity of the transglutaminase band in the cells incubated for 0.5 h is no different from control. However, cells exposed to retinoic acid for 1 and 2 h showed a marked increase in the intensity of the transglutaminase band. Denitometric scanning of the band at 2 h indicated that the rate of enzyme synthesis was increased at least 7-fold after 2 h of exposure to retinoic acid.

As an independent means of analysis, the induction of tissue transglutaminase was followed by observing the time course of accumulation of enzyme activity in retinoic acid- or serum-treated cells. Fig. 5 shows the accumulation of transglutaminase activity in macrophages exposed to fresh mouse serum (C) or delipidized serum containing 1 nM (□) or 1 μM (●) trans-retinoic acid. In the cells exposed to fresh serum, the enzyme activity was increased 60–90 min after exposure of the cells to serum. The same delay was seen in cells exposed to either 1 nM or 1 μM retinoic acid. It is also apparent from

**Fig. 3. Concentration dependence of retinoid induction of tissue transglutaminase.** A. transglutaminase activity of macrophages cultured for 20 h in RPMI plus 20% delipidized mouse serum and varying concentrations of trans-retinoic acid (RA). B. transglutaminase activity of macrophages cultured for 20 h in varying concentrations of trans-retinoic acid (T-RA, ●), cis-retinoic acid (C-RA, ○), and retinol (ROH, △) in media plus 20% delipidized mouse serum.
Retinoic Acid Induction of Macrophage Transglutaminase

Fig. 4. Effect of retinoic acid on the rate of synthesis of tissue transglutaminase. Macrophages were preincubated for 60 min in media plus 10% delipidized mouse serum before 1 μM retinoic acid was added. The rate of synthesis of tissue transglutaminase was measured by pulse-labeling the cells with [35S]methionine followed by immunoprecipitation of the transglutaminase and autoradiography as described under "Experimental Procedures." Lane 1 shows the immunoprecipitate obtained with preimmune IgG. Lane 2 shows the immunoprecipitate of cells cultured in delipidized serum prior to the addition of retinoic acid. Lane 3 shows the immunoprecipitate of cells pulse-labeled 30 min following the addition of retinoic acid. Lane 4 shows the immunoprecipitate 60 min following addition of retinoic acid and lane 5 shows the immunoprecipitate 120 min following the addition of retinoic acid to the cells.

Fig. 5 that the effect of different concentrations of retinoic acid is to alter the rate of accumulation of the enzyme; once induced, the enzyme continued to accumulate for at least 20 h. The effect of retinoic acid on transglutaminase expression can be completely blocked by actinomycin D. Table II shows the effect of 0.2 μg/ml actinomycin D on retinoic acid-induced expression of transglutaminase activity in cultured macrophages.

To evaluate the specificity of the retinoic acid-induced expression of tissue transglutaminase, cells were metabolically labeled with [35S]-amino acids while being exposed to delipidized serum in the presence or absence of retinoic acid. The cells were then solubilized and fractionated by SDS-gel electrophoresis and the protein bands were detected by fluorography (Fig. 6). The only major difference in the pattern of polypeptides between the control (lane 1) and the retinoic acid-treated cells (lane 2) was a prominent band that co-electrophoresed with purified tissue transglutaminase (acute arrow in Fig. 6). The identity of this band as tissue transglutaminase was confirmed by immunospecific precipitation by an anti-transglutaminase antibody (lane 4) and not by preimmune IgG (lane 3). The only other consistent difference observed in the polypeptides of control and retinoid-treated cells is a band at 35,000 Da (shown by the blunt arrow in Fig. 6) that appears to be consistently increased in cells incubated in the presence of trans-retinoic acid. The identity of this band is unknown to us.

We have recently reported that phagocytosis suppresses serum-induced expression of tissue transglutaminase (Mehta et al., 1984). To see if phagocytosis also reduced the ability of retinoic acid to induce expression of tissue transglutaminase, macrophages were preincubated with IgG-coated liposomes and the induction of transglutaminase activity was compared with untreated cells (Table III). Treatment of macrophages with liposomes had little effect on the basal levels of transglutaminase activity but it reduced by 70% the accumulation of the enzyme that occurred in response to retinoic acid and delipidized serum.

Role of Serum in Retinoic Acid-induced Expression of Tissue Transglutaminase—Two proteins are present in serum that can bind retinoic acid, serum albumin and SRBP (Horowitz and Heller, 1973; Smith et al., 1973). The experiments shown in Fig. 2 and Table I indicated that albumin was much less effective than delipidized serum in augmenting retinoic acid-induced expression of tissue transglutaminase. Therefore, we
Retinoic Acid Induction of Macrophage Transglutaminase

FIG. 6. Specificity of retinoic acid-induced expression of macrophage tissue transglutaminase. Peritoneal macrophages were cultured in RPMI 1640 media containing 14C-labeled amino acids (10 µCi/ml) and either 10% delipidized mouse serum alone or 10% delipidized serum plus 1 µM trans-retinoic acid. After 24 h, the cells were washed and lysed with Triton X-100. Lane 1 contains a portion of the Triton extract from cells exposed to delipidized serum alone. Lane 2 contains cell extracts exposed to delipidized serum plus retinoic acid. Retinoic acid-treated cell extracts were also immunoprecipitated with preimmune IgG (lane 3) or anti-transglutaminase antibody (lane 4) as described under "Experimental Procedures." All samples were fractionated on a 6.5% discontinuous SDS-polyacrylamide gel and cell proteins were identified by fluorography. The upper arrow marks the mobility of purified tissue transglutaminase. The lower arrow marks the unidentified 35,000-Da protein that also appears to be induced by retinoic acid.

TABLE III

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<th>Culture conditions*</th>
<th>Transglutaminase activity</th>
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<tr>
<td>Untreated cells, RPMI alone</td>
<td>79 ± 21</td>
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<tr>
<td>Untreated cells, RPMI, 20% delipidized mouse serum, 10 nM retinoic acid</td>
<td>537 ± 76</td>
</tr>
<tr>
<td>MLV-treated cells, RPMI alone</td>
<td>119 ± 27</td>
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<tr>
<td>MLV-treated cells, RPMI, 20% delipidized mouse serum, 10 nM retinoic acid</td>
<td>249 ± 25</td>
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* Monolayers of macrophages were pre-incubated with RPMI alone or RPMI containing 500 µg/ml IgG-coated multilamellar vesicles (MLV) for 60 min. The cells were then washed extensively and then cultured for 4 h in either RPMI media alone or RPMI media containing 20% delipidized mouse serum and 10 nM retinoic acid. The values shown represent the mean and standard deviation of replicate determinations.

evaluated the role of SRBP in the induction of tissue transglutaminase by using an antibody to selectively remove it from delipidized mouse serum. We then compared the ability of untreated or SRBP-depleted serum to promote retinoic acid-induced expression of tissue transglutaminase. Delipidized mouse serum was incubated with either normal rabbit globulin or rabbit anti-human SRBP and immune complexes were precipitated by addition of washed, heat-killed S. aureus. To ensure that this procedure was effective in removing SRBP from the serum, we fractionated an aliquot of the serum by electrophoresis on a 15% polyacrylamide gel and identified the SRBP by Western blot analysis with the anti-SRBP antibody and 125I-labeled protein A (Fig. 7). Comparison of control and antibody-treated serum (lanes 1 and 2) showed that the immunoprecipitation procedure completely removed the SRBP (shown by the arrow in Fig. 7) from the delipidized mouse serum.

We next compared the ability of control and anti-SRBP-treated serum to promote retinoic acid-induced expression of transglutaminase. Table IV shows the results of two separate experiments. In each instance, depletion of the SRBP from the delipidized serum completely abolished its ability to promote retinoic acid-induced expression of transglutaminase.

Serum from different sources may have varying intrinsic activity in inducing expression of tissue transglutaminase (Fig. 8 and Murtaugh et al., 1983). The experiment shown in Fig. 8 compared the levels of transglutaminase activity in macrophages cultured in fresh mouse serum with the activity in cells cultured in the same concentration of an inactive batch of mouse serum purchased from a commercial supplier, fetal calf serum, and human serum. Addition of retinoic acid to these sera produced only a small increase in the induction of the enzyme. If, however, these sera were first delipidized
and then recombined with retinoic acid, there was a large increase in their ability to promote the accumulation of the enzyme (Fig. 8). These studies indicate that exogenous retinoic acid is much more effective when recombined with delipidized serum than when it is added to intact serum.

**DISCUSSION**

Reconstitution of the Transglutaminase-inducing Activity of Delipidized Serum with trans-Retinoic Acid—The starting point for our studies was the observation that delipidization of fresh mouse serum completely destroyed its ability to induce the expression of tissue transglutaminase. The delipidization procedure we used quantitatively extracts serum retinoids (Fuchs and Green, 1981). Since retinoids have been reported to alter the transglutaminase activity of cultured epidermal and melanoma cells (Yuspa et al., 1980; Scott et al., 1982; Yuspa et al., 1982) we included them in our initial survey of compounds that might reconstitute the transglutaminase-inducing activity of mouse serum. We found that trans-retinoic acid restored the ability of delipidized serum to induce the enzyme and it was the only compound we tested that showed this activity. Not only did trans-retinoic acid induce the enzyme but it did so at very low levels. The concentration of retinoic acid in normal human plasma is 3–10 nM (De Ruyter et al., 1979) and may be as high as 100 nM in rodent plasma (Shidoji and Hosoya, 1980). Addition of retinoic acid in this concentration range to delipidized serum produced a large induction of tissue transglutaminase, suggesting that there is sufficient retinoic acid in fresh serum or plasma to totally account for the induction of the enzyme.

The induction of tissue transglutaminase by fresh serum showed several distinctive features. The induction itself developed very rapidly after exposure of the cells to serum and was due to an increased rate of enzyme synthesis (Murtaugh et al., 1983). Furthermore, the induction of transglutaminase was blocked by pretreatment of the cells with inhibitors of RNA synthesis such as actinomycin D (Murtaugh et al., 1983). Third, the phagocytosis of opsonized particles by the macrophages markedly blunted their response to serum (Mehta et al., 1984). Each of these features was reproduced by the re-
addition of retinoic acid to delipidized serum. The time course for the induction of the enzyme (Fig. 5) showed the same 90-min time lag for both intact and reconstituted serum. The pulse-labeling studies showed that retinoic acid produced an increased rate of enzyme synthesis and, like intact serum, this effect was blocked with actinomycin D. Last, phagocytosis suppressed the response of the cells to reconstituted serum as effectively as it blocked the response to intact serum. As best as we can determine, adding retinoic acid back to delipidized mouse serum completely reconstitutes its ability to alter the expression of tissue transglutaminase. Since the amounts of retinoic acid added back are comparable to those found in intact serum, we believe it very likely that retinoic acid is one of the components, if not the only component, of serum responsible for the induction of tissue transglutaminase in cultured mouse peritoneal macrophages.

**SRBP as a Cofactor for Retinoic Acid-induced Expression of Tissue Transglutaminase**—Retinoic acid added to serum-free media was able to induce a small but significant increase in the level of tissue transglutaminase. Retinoic acid was, however, much more effective and much more potent if it was first recombined with delipidized serum. This observation suggested to us that serum contained a second factor that increased the ability of retinoic acid to induce tissue transglutaminase. Retinoic acid binds tightly to serum albumin (Smith et al., 1973; Sani et al., 1978); therefore, we tested the effects of albumin on retinoic acid-induced expression of transglutaminase. The results of these experiments were uniformly negative; retinoic acid bound to albumin did not regulate the expression of macrophage transglutaminase. This finding may be of considerable biological significance. Exogenous retinoic acid administered to animals in the diet or by injection is transported in the plasma bound to albumin (Smith et al., 1973). However, retinoic acid bound to albumin is not taken up by the cells of the pigmented epithelium of the retina (Chen and Heller, 1978). These studies and our own findings suggest that nanomolar concentrations of retinoic acid bound to albumin may not be biologically active and that under physiological conditions dietary retinoic acid may not be an important contributor to retinoic acid-regulated gene expression.

Serum also contains a specific retinoid-binding protein, SRBP (for reviews, see Rask et al., 1986; Goodman, 1982). In normal serum, it is present entirely as a holoprotein containing tightly bound retinoid (Kanai et al., 1968; Peterson, 1971; Goodman and Raz, 1972). In intact plasma, the predominant retinoid bound to SRBP is retinol. This retinol is readily extracted with the organic solvents used to delipidize serum (Futterman and Heller, 1972; Goodman and Raz, 1972; Fuchs and Green, 1981). Apo-SRBP binds both retinol and retinoic acid with high affinity (Horowitz and Heller, 1973; Cogan et al., 1976). Since apo-SRBP binds retinoic acid avidly and since retinoic acid bound to SRBP can be rapidly taken up by at least one cell type, the pigmented epithelium of the retina (Chen and Heller, 1978), we examined the possibility that the apo-SRBP in delipidized serum was responsible for the observed potentiation of retinoic acid action. We depleted all the apo-SRBP from delipidized serum by immunoprecipitating it with a specific antibody and found that the SRBP-depleted serum was incapable of enhancing retinoic acid-induced expression of the tissue transglutaminase. We believe that when retinoic acid is added to delipidized serum it binds to apo-SRBP and forms a complex (retinoic acid-SRBP) that is much more effective than free retinoic acid in inducing expression of macrophage transglutaminase. This suggests that the biologically active form of retinoic acid in serum, at least in terms of macrophage gene expression, is the complex of retinoic acid bound to SRBP. The concentrations of this complex in normal serum and the factors that may regulate its expression and activity are entirely unknown.

The preceding studies suggest that low levels of retinoic acid need to be complexed to SRBP for biological activity, at least in terms of macrophage gene expression. Since intact serum presumably contains holo-SRBP as the predominant SRBP species, it should be less active than delipidized serum in potentiating the effects of exogenous retinoic acid. The results of the experiments shown in Fig. 8 support this proposition. In each instance, delipidized serum was more effective than intact serum in supporting retinoic acid-induced expression of macrophage transglutaminase. Thus, the addition of retinoic acid to media containing serum, particularly fetal calf serum, is a relatively ineffective way to deliver the retinoid to susceptible cells. At least in the case of macrophages, retinoic acid is much more effective when added to delipidized rather than intact serum.

Serum retinol-binding protein is thought to be responsible for the intravascular transport of retinol and its delivery to specific target tissues (Kanai et al., 1968; Peterson, 1971; Heller, 1975; Rask and Peterson, 1976; Ganguly et al., 1980; Rask et al., 1980). Receptors for the SRBP present on the surface of target cells would account for the specificity of the delivery process (Heller, 1975; Rask and Peterson, 1976). The binding of the retinol-SRBP complex to cell surface receptors apparently facilitates the delivery of the retinol into the interior of the cell (Heller, 1975; Bok and Heller, 1976; Rask and Peterson, 1976; Jones and Heller, 1980). Similarly, retinoic acid bound to SRBP is preferentially delivered to cells of the pigmented epithelium of the retina (Chen and Heller, 1978). This suggests that the cellular uptake of retinoic acid may be accomplished by a receptor-mediated pathway very similar to that used for the uptake of retinol. Such a pathway might account for our finding that physiological concentrations of retinoic acid (1–100 nM) were only capable of inducing transglutaminase gene expression if SRBP was also present. If the macrophage contains cell surface SRBP receptors similar to those described on other cells, then they may participate in the receptor-mediated delivery of low levels of retinoic acid to the cell. At pharmacologic levels of retinoic acid (100–1000 nM), such a receptor-mediated delivery system may become irrelevant as the retinoid diffuses directly into the cell. This may explain why high levels of retinoic acid can increase transglutaminase gene expression in the absence of serum proteins. The presence of both receptor-mediated and nonselective pathways for the entry of retinoic acid into cells would account for the wide variations in the concentrations of retinoic acid required to elicit biological effects in different types of cultured cells (Strickland and Mahdavi, 1978; Breitman et al., 1980; Lacroix et al., 1980; Linder et al., 1981; Thein and Lotan, 1982; Yuspa et al., 1982; Lehtonen et al., 1983).

**Retinoic Acid and the Expression of Macrophage Transglutaminase**—The macrophage is not the only cell known to respond to retinoic acid by alterations in the levels of transglutaminase activity. Epidermal cells treated both with retinoic acid and phorbol esters showed marked increases in transglutaminase activity (Yuspa et al., 1980, 1982). There are, however, important differences between the expression of epidermal cell and macrophage transglutaminase. Epidermal cells contain a unique transglutaminase, epidermal transglutaminase, that is biochemically and immunohistochemically distinct from tissue transglutaminase (Ogawa and Goldsmith, 1976) and it is presumably this enzyme whose activity is increased by retinoic acid. It is not clear whether the increase
in epidermal transglutaminase is a direct reflection of retinoic acid-induced alterations in epidermal transglutaminase gene expression or an activation of pre-existing enzyme. Alterations of enzyme activity only appear several hours after the addition of retinoic acid (Yuspa et al., 1982) and can be blocked by specific protease inhibitors (Kawamura et al., 1983). These findings suggest that alterations in transglutaminase activity in epidermal cells in response to retinoic acid may be a more indirect process than the induction of tissue transglutaminase in peritoneal macrophages. In spite of these differences, it still is striking that, in two specialized target cells, retinoic acid induces specific increases in transglutaminase activity.

It also has been reported that retinoic acid can alter the transglutaminase activity of mouse melanoma cells (Scott et al., 1982). This response, however, is quite different from the response of macrophages to the retinoids. In melanoma cells, retinoic acid induced a slight increase in the cellular response to melanocyte-stimulating hormone and the effect was very transient, being detected in 60 min and gone completely in 180 min. Furthermore, this effect was seen only with very large doses (80 μM) of retinoic acid, suggesting the phenomenon is quite different from retinoic acid-induced expression of macrophage transglutaminase.

Our studies do not define precisely the molecular events involved in the retinoic acid-induced accumulation of transglutaminase in macrophages. But they do give some hints as to the types of processes that are involved. It is apparent from our results that treatment of macrophages with retinoic acid induced a large and very selective increase in the amounts of tissue transglutaminase in the cells. The high degree of specificity in this induction and its inhibition by actinomycin D suggest but do not prove that this response is due to increased transglutaminase gene expression.

There is considerable evidence that altered gene expression is an early component in retinoic acid-induced cellular differentiation (for a review, see Sporn and Roberts, 1983). Using tissue transglutaminase as a specific probe, we can say that within 90 min of exposure of a target cell to retinoic acid there are dramatic alterations in the expression of at least one particular gene, tissue transglutaminase. There are likely to be other proteins whose expression is altered by retinoic acid but they are present at levels too low to be detected with the analytical procedures we have used. The rapidity of this response, which is comparable to the effects of steroid hormones on gene expression, suggests that there must be a relatively direct relay of information from the appearance of retinoic acid outside the cell to the activation of specific genes. Whether this involves indirect processes such as protein phosphorylations or the direct translocation of retinoic acid to the nucleus is a subject of current investigation in our laboratory.

We do not know the physiological significance of the induction of tissue transglutaminase in the macrophage. The accumulations of the enzyme we have observed in response to retinoic acid in vitro are no greater than the accumulations of the enzyme found in fully activated macrophages. In these cells, tissue transglutaminase can account for close to 1% of cellular protein. It has been suggested that these high levels of tissue transglutaminase may contribute to the enormous phagocytic capacity of these cells (Fesus, 1982), but the role of the enzyme in phagocytosis or any other functions of the macrophage remains to be established. In spite of our uncertainty as to the precise physiological role of transglutaminases in the macrophage, we believe that the retinoic acid-induced regulation of this enzyme in macrophages will be a powerful tool for understanding the molecular events involved in retinoid control of gene expression.

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