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Cell-mediated lysis of lipid vesicles containing eye muscle protein: Implications regarding pathogenesis of Graves ophthalmopathy*

(T and K lymphocytes/thyroglobulin/antithyroglobulin/cell membranes/autoimmunity)

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ABSTRACT **We prepared artificial vesicles that are lysed upon cell-mediated immunological attack by human lymphocytes. These vesicles are made from a mixture of dimyristoyl lecithin, dipalmitoyl lecithin, and cholesterol, have eye muscle membrane protein (EMP) inserted into the bilayer wall, and contain intravesicular ^{99m}Tc marker. Injury to the vesicular membrane was assessed by measurement of ^{99m}Tc release. Thyroglobulin (Tg) and Tg–anti-Tg complex (TgA) bind to EMP-vesicles to an extent equal to or greater than to native eye muscle membranes *in vitro*; this binding requires the presence of normal human IgG. The role of Tg, TgA, IgG, and peripheral blood lymphocytes in altering membrane permeability was analyzed. Incubation of vesicles for up to 3 hr alone, with added IgG alone, or with further addition of Tg or TgA did not result in ^{99m}Tc release. Addition of lymphocytes from normal donors to the above four preparations showed release in the presence of TgA. Lymphocytes from each of eight patients with Graves ophthalmopathy caused release not only in the presence of TgA, but also in the presence of Tg. Separation of a patient's lymphocytes into high- and low-affinity rosette-formers (T and K cells, respectively) showed that cell-mediated vesicle lysis in the presence of TgA was greater with K cells than with T cells, while vesicle lysis in the presence of Tg was greater with T cells than with K cells. Vesicles made with inserted Tg but lacking EMP were not lysed by such T cells. Lymphocytes failed to induce permeability changes in vesicles containing other inserted proteins obtained from human nonextraocular muscle, liver, spleen, or adrenal, even if Tg or TgA were present. The results support the concept that muscle cell damage in Graves ophthalmopathy is immunological, cell-mediated, and of two types: (*i*) K lymphocytes reacting to immune complex, TgA, on the eye muscle cell surface (i.e., antibody-dependent cytotoxicity) and (*ii*) sensitized T lymphocytes reacting to Tg on the eye muscle cell surface. An antigenic role for EMP is possible, but has not been unequivocally proven.**

Graves ophthalmopathy is an eye disorder confined to human beings and characterized by symptoms that may include proptosis, excessive lacrimation, periorbital fullness, conjunctival edema, diplopia, and sight loss. Gross enlargement of the extraocular muscles is characteristic. Lymphocytic infiltration of these muscles and various degrees of muscle cell damage are observed on microscopy. The muscular damage may be the result of a cell-mediated cytotoxic reaction (2–7), but the evidence is circumstantial: the orbit and the thyroid share common lymphatic drainage channels, and substances deposited in or released from the thyroid gland may travel in a cephalad direction (2); a normal thyroid gland secretory product, thyroglobulin (Tg), and its corresponding immune complex (TgA) have an affinity for eye muscle membranes (3, 7); autoantibodies to thyroid tissue or products—e.g., anti-(thyroid) microsomal antibody and anti-Tg—are often detectable in the serum (8); orbital tissue homogenate will inhibit the migration *in vitro* of leukocytes obtained from patients with Graves ophthalmopathy (4, 5); and Tg associated with orbital muscle is the component in orbital tissue responsible for the latter reaction, and Tg determinants have been detected on normal human orbital muscle fibers, but not on those of other skeletal muscle (6).

The inaccessibility and unavailability of living human eye muscle for direct experimentation led us to study the mechanism of muscle injury by developing an artificial lipid vesicle system incorporating eye muscle protein into the bilayer wall and enclosing a radioactive marker that might be released if the vesicles were lysed by a natural immunologic mechanism. Using this system we hoped to determine the role, if any, of eye muscle protein, added Tg, TgA, normal human IgG, and lymphocytes in experimental vesicle lysis, and, by analogy, in the muscle cell damage observed in patients with Graves ophthalmopathy.

In this report we describe the demonstration of cell-mediated lipid vesicle lysis with lymphocytes obtained from healthy human subjects and from patients with Graves ophthalmopathy, delineate some of the physicochemical conditions required for such lysis, and report that the conditions for lysis with lymphocytes from patients with Graves ophthalmopathy differ from those of patients with Graves disease without ophthalmopathy, those with several other thyroid or eye diseases, and normal healthy controls. These studies indicate that eye muscle protein is a necessary ingredient of the vesicular wall and that, with lymphocytes from patients with ophthalmopathy, lysis always occurs in the presence of Tg or its immune complex, TgA, while vesicle lysis with lymphocytes from other subjects occurs only in the presence of TgA.

MATERIALS AND METHODS

Experimental Subjects. Six healthy subjects and 14 patients with thyroid or eye disease attending the Stanford Nuclear Medicine Clinic donated 10–20 ml of venous blood for subsequent separation and testing of their lymphocytes. Diagnoses were based on clinical assessment and history, symptoms and signs, plus measurement by radioimmunoassay of serum thyroxine, triiodothyronine, anti-(thyroid) microsomal antibody (8), and anti-Tg (8). In two patients with thyroid carcinoma and one with Hashimoto thyroiditis, surgical specimens were available for definitive microscopic diagnoses. Graves ophthalmopathy was diagnosed in eight patients by standard criteria (9); each had a present or past history of thyroid disease; seven

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Abbreviations: Tg, thyroglobulin; TgA, Tg–anti-Tg complex; ^{125}I-Tg and ^{125}I-TgA, ^{125}I-labeled Tg and TgA, respectively; EMP, eye muscle membrane protein.

* Portions of this work have been presented in preliminary form [ref. 1 and Kriss, J. P., Mehdi, S. Q. & Sheng, R. (1978) in *Program of the 54th Meeting, American Thyroid Association, Portland, OR*, p. T-1].

had received [^{131}I]iodide therapy; one was taking propylthiouracil. Computed tomographic scans of the orbit were useful in corroborating the diagnosis of Graves ophthalmopathy and distinguishing the one patient with orbital pseudotumor (10, 11); the latter patient lacked a history of thyroid disease and all above serum tests were normal. The results of the above serum tests had to be completely normal before the six healthy donors were considered to be normal controls.

Preparation of Membranes. Human eye muscle and other tissues were obtained post mortem, kept in liquid nitrogen, and thawed in the refrigerator at 0–4°C overnight before use. Freshly excised bovine eyes and attached muscles were transported on ice from a local abattoir, and the extraocular muscles were dissected out and processed immediately.

Plasma membranes were prepared from tissue homogenates by sucrose density gradient centrifugation (12). The tissue was minced finely and homogenized in 3–5 vol (wt/vol) of 10 mM Tris·HCl, pH 7.4/30 mM NaCl/1 mM $MgCl_2$/5 μM phenylmethylsulfonyl fluoride/0.25 M sucrose. After two 5-sec treatments at setting no. 8 of a Polytron homogenizer, the homogenate was filtered through four layers of cheese cloth, layered over 10 ml of 1.31 M (41%) sucrose in the same buffer, and centrifuged for 2 hr at 95,000 × *g* in a Beckman SW 27 rotor. Membranes were harvested from the interface of sucrose solution and the homogenate and were pelleted by centrifugation at 105,000 × *g* for 1 hr. After two washes by centrifugation and resuspension, the pellets were stored under liquid nitrogen until used.

Preparation of Tg and TgA. Human Tg was prepared by gel filtration of human thyroid homogenates as described (13). Human anti-Tg was prepared from the serum of a patient with Hashimoto thyroiditis by affinity chromatography (14) on Sepharose 4B-Tg. The original serum contained 3600 units/mg of protein (8); the purified product contained 97,800 units/mg of protein. TgA was prepared as described (7).

Preparation of Lymphocytes. Human peripheral blood lymphocytes were prepared by centrifuging anticoagulated blood in Ficoll/Hypaque (15), harvesting the cells at the interface of the two solutions, and suspending them in Hanks' balanced salt solution (pH 7.4) before use. B lymphocytes and high- and low-affinity E rosette-forming T cells (T and K cells, respectively) were prepared as described (16).

Preparation of Vesicles and Insertion of Protein and Marker. Pure phospholipid vesicles were prepared by dissolution of 25 mol % dimyristoyl lecithin, 45 mol % dipalmitoyl lecithin, and 30 mol % cholesterol in chloroform/methanol, 2:1 (vol/vol). In early trials 14 mol % phosphatidylserine was also present; this ingredient was found to be unnecessary. The mixture was dried under reduced pressure at 55°C as a film on the inside of the flask and then hydrated with 2 ml of Tris·HCl (pH 7.4). The mixture was kept under nitrogen and after it was thoroughly mixed in a Vortex mixer, it was sonicated at 55°C for 1 hr (microtip, Bronwill-Biosonik, power setting 3). Membrane proteins were obtained from membranes by treatment with 1% deoxycholate and harvested after sucrose gradient centrifugation (17). Membrane proteins or Tg was inserted into the artificial lipid vesicles by following exactly the described method (18). Protein was measured by the method of Lowry *et al.* (17), phospholipids were estimated by measurement of organic phosphorus (19) and by thin-layer chromatography (18), the cholesterol was measured with the cholesterol kit from Abbott. The protein content of the vesicles was 59 ± 5% (wt/wt); cholesterol content was approximately 30 mol %.

The intravesicular marker $^{99m}TcO_4^-$ was incorporated as follows. Vesicles were incubated with 10 mCi of $^{99m}TcO_4^-$ (1 Ci = 3.7 × 10^{10} Bq) for 2–3 hr at room temperature. During the last hour they were sonicated in a water bath. The sonicated suspension was applied to a 1 × 10 cm column of Sepharose 2B in Hanks' solution, and the vesicles were eluted with the same solution. They were identified by their opalescence and ^{99m}Tc content, as measured in a well-type scintillation counter.

Incubation Ingredients. These consisted of reconstituted lipid vesicles containing eye muscle protein (EMP) and intravesicular $^{99m}TcO_4^-$, added Tg or TgA, and peripheral blood lymphocytes from an individual donor, suspended in Hanks' solution. Control incubation mixtures included the following: (*i*) pure lipid vesicles, (*ii*) vesicles containing amounts of membrane protein equivalent to EMP but obtained from other human tissue sources (liver, spleen, adrenal, cheek or gluteal skeletal muscle, thyroid, and kidney), (*iii*) vesicles containing EMP but made of only a single phospholipid (dipalmitoyl lecithin), (*iv*) vesicles with incorporated Tg instead of membrane protein, (*v*) Tg and TgA omitted, and (*vi*) lymphocytes omitted. Before addition, lymphocytes were kept in polystyrene tubes at 37°C in Hanks' solution. The vesicles were recognizable as tiny spherical bodies approximately 1–2 μm in size; they and the lymphocytes were counted in a standard hemocytometer chamber. A typical incubation mixture contained 5 × 10^6 vesicles, 5 × 10^5 lymphocytes, and 1 μg of Tg or TgA in a total volume of 400 μl.

Measurement of Vesicle Lysis. The various vesicle preparations containing intravesicular $^{99m}TcO_4^-$ were mixed in a dialysis bag in 0.5–1.0 ml of Hanks' solution with or without other added ingredients, such as lymphocytes, Tg, TgA, and IgG, and the dialysis bag in turn was placed in a conical flask containing 20 ml of the same solution. The dialysis bag was able to turn freely in the dialysate. The mixture was kept at 37°C. At various intervals up to 2 or more hours as indicated, a 1-ml aliquot of dialysate was removed. At the end of the experiment, the radioactivity in the dialysis bag and all specimens was determined and appropriate corrections were made for radioactive decay and volume change. The percentage of the bag radioactivity that leaked into the dialysate was then calculated for each sampling interval.

Materials. Cholesterol, dimyristoyl lecithin, and dipalmitoyl lecithin were from Supelco (Bellefonte, PA); heat-inactivated fetal calf serum was from GIBCO; Na^{125}I was from New England Nuclear; and $^{99m}TcO_4^-$ was from a ^{99}Mo generator supplied by New England Nuclear.

Proteins were iodinated by a modification of the method of Hunter and Greenwood (20) as described (12).

Binding of ^{125}I-Labeled Tg (^{125}I-Tg) and TgA (^{125}I-TgA). Pure membranes, pure lipid vesicles, or EMP–vesicles were incubated with ^{125}I-Tg or ^{125}I-TgA at 37°C for 1 hr. Aliquots were transferred to microfuge tubes containing 100 μl of 5% bovine serum albumin and centrifuged for 5 min at 10,000 × *g*. The tubes were inverted and cut just above the vesicle pellet, and both free radioactivity and that bound to vesicles were measured.

RESULTS

Binding of ^{125}I-Tg and ^{125}I-TgA to bovine eye muscle membranes, pure lipid vesicles, and EMP–vesicles was measured after incubation for 1 hr at 37°C in Tris/EDTA buffer (7) in the presence and absence of 1 mg of normal human IgG per 100 ml (Table 1). Pure lipid vesicles did not bind Tg or TgA. The magnitude of binding to native eye muscle membranes equalled or exceeded that previously reported (7). Binding of both Tg and TgA to EMP–vesicles was better than that observed with the native eye muscle membranes of comparable protein content; the presence of normal human IgG was required for this binding. TgA binding was approximately 3-fold higher than

Table 1. Binding of ^{125}I-Tg and ^{125}I-TgA to bovine eye muscle membranes and artificial vesicles

Preparation	Normal human IgG	^{125}I-Tg, % bound	^{125}I-TgA, % bound
Eye muscle membranes	−	3	6
	+	17	40
Vesicles, lipid only	−	3	3
	+	0	0
Vesicles–EMP	−	3	3
	+	23	67

^{125}I-Tg or ^{125}I-TgA (≈2 ng; 100,000 cpm) was incubated with eye muscle membranes (25 μg of protein), artificial lipid (25 μg) vesicles, or vesicles containing EMP (25 μg) in 200 μl of 10 mM Tris·HCl/1 mM EDTA at pH 7.4. After incubation at 37°C for 1 hr in the presence or absence of 10 μg of normal human IgG, 30-μl aliquots were removed in duplicate, layered over 300 μl of 6% bovine serum albumin, and centrifuged for 2 min at 10,000 × *g* in a microfuge. Radioactivity in the pellet and the supernatant was measured separately. Results are average of duplicates. Bound radioactivity is expressed as a percentage of the total radioactivity added.

that of Tg for both native eye muscle and vesicle–EMP preparations.

Rate of Release of Intravesicular Marker under Control Conditions. The release of intravesicular ^{99m}Tc from pure lipid vesicles was first examined. Eight to 10% of the radioactivity was measured in the dialysate during the initial 20 min of incubation. After this, only a small increase was observed for the following 3–6 hr. Separate addition of normal human IgG, Tg, TgA, or lymphocytes alone did not significantly alter the release pattern.

Similar observations were made with the separate addition of each of the above constituents to Tg–vesicles or to EMP–vesicles alone. In fact, the separate addition of Tg, TgA, or lymphocytes to EMP–vesicles had a slight protective effect which lowered the release of the intravesicular marker to about 6%. If the release of the marker under these "control" conditions was 20% or more of that which could be released by Triton X-100 (normally 70–85% of the total intravesicular radioactivity), then the results of the experiment were discarded, since such a result indicated that the vesicles were unstable and that release resulting from vesicle lysis would not be clearly distinguishable from normal release.

Lymphocyte-Mediated Lysis of EMP–Vesicles. For each set of incubation mixtures containing the full complement of ingredients (i.e., EMP–vesicles, Tg, or TgA and lymphocytes), the "control" rate of leakage of the intravesicular marker was measured in the absence of lymphocytes. When EMP–vesicles were incubated with Tg or TgA and lymphocytes from a normal donor, an increase in ^{99m}Tc release compared to control was observed only when TgA was present (Fig. 1). This type of response was observed with five of the healthy donors, two patients with Graves disease without ophthalmopathy, two patients with thyroid cancer previously treated by surgery and [^{131}I]iodide, a patient with Hashimoto thyroiditis, and a patient with orbital pseudotumor. The mean (± SEM) experimental/control release ratio at 90 min for these subjects was 3.7 ± 0.3 %. When lymphocytes from a patient with Graves ophthalmopathy were incubated with EMP–vesicles, lysis was observed not only in the presence of TgA, but also in the presence of Tg, although the magnitude of the latter was slightly lower (Fig. 2). This type of response was observed in each of the eight patients tested who had Graves ophthalmopathy. For this group, the mean (± SEM) experimental/control release ratio at 90 min

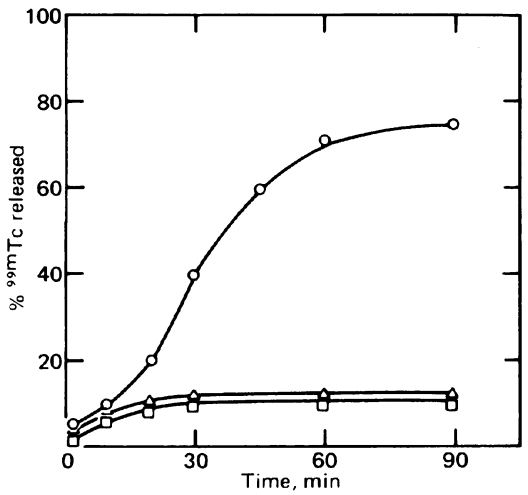

FIG. 1. Vesicle lysis by lymphocytes from normal subject. EMP–vesicles (5 × 10^6) containing $^{99m}TcO_4^-$ were incubated in 400 μl of Hanks' solution with Tg (1 μg) or TgA (1 μg) at 37°C for 1 hr. The mixture was put in dialysis tubing, and lymphocytes (5 × 10^5) in 400 μl of Hanks' solution were added. The tubing was placed in a flask containing 20 ml of Hanks' solution and the incubation was continued for 90 min. Samples (1 ml) of dialysate were withdrawn at intervals for measurement of radioactivity. Corrections were made for ^{99m}Tc decay and volume change. Radioactivity released from the vesicles by lymphocytes from a normal individual in the presence of Tg (△) or TgA (○) is shown. That released from the vesicles alone was used as a control (□).

in the presence of TgA and Tg was 3.9 ± 0.3 % and 2.8 ± 0.1 %, respectively. The difference between the latter values was significant ($P < 0.005$). The second type of response was also observed in one healthy subject.

However, when vesicles containing Tg in the lipid bilayer, but not containing EMP, were incubated with lymphocytes from patients with Graves ophthalmopathy or from control subjects, no marker release was observed under any of the conditions described above.

Effect of High-Affinity E-Rosette-Forming Cells (T Lymphocytes) and Low-Affinity E-Rosette-Forming Cells (K Lymphocytes) on Tg- and TgA-Mediated Release of Intravesicular Marker from EMP–Vesicles. Human T cells can be separated into two subsets on the basis of their relative affinity

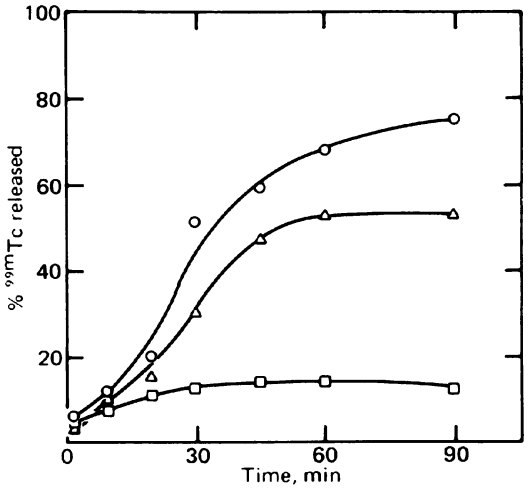

FIG. 2. Experimental procedure was similar to that for Fig. 1. Human EMP–vesicles were incubated alone (□) or with lymphocytes from a patient with Graves ophthalmopathy in the presence of either Tg (△) or TgA (○). The responses were replicated in this patient on four separate occasions.

for sheep erythrocytes (16). High-affinity E-rosette-forming cells contain few antibody-dependent cytotoxic cells (ADCC-K cells), whereas low-affinity E-rosette-forming cells, requiring cool temperatures and excess sheep erythrocytes, were highly enriched for antibody-dependent cytotoxic activity. For convenience the high- and low-affinity E-rosette-forming cells are called T and K cells, respectively.

T and K cells were prepared from peripheral blood lymphocytes of a patient with ophthalmopathy. The unseparated lymphocytes from this patient showed enhanced marker release in the presence of both TgA and Tg. As shown in Fig. 3, ^{99m}Tc released from EMP-vesicles by T cells in the presence of Tg alone was greater than in the presence of TgA. Conversely, the response to K cells was much smaller in the presence of Tg than TgA. This evidence suggests that cell preparations enriched in T cells required only Tg to provoke vesicle lysis, while the preparation enriched with K cells (antibody-dependent cytotoxic cells) required TgA to elicit marker release from the vesicles.

Effect of Insertion of Other Membrane Proteins. Membrane proteins obtained from a number of different human tissues were inserted into vesicles in the same manner and quantity as the preparation of EMP-vesicles: liver, spleen, adrenal, gluteal and cheek skeletal muscle, kidney, and thyroid. Each of these vesicles was tested in the same manner as EMP-vesicles by using lymphocytes obtained from six different subjects, two with Graves ophthalmopathy, two with Graves disease without ophthalmopathy, and two healthy controls. Vesicles containing liver, spleen, adrenal, or nonorbital muscle membrane proteins were not lysed under any test conditions. Vesicles containing kidney and thyroid membrane protein were not lysed under any conditions by five of the six subjects. The single exception for thyroid membrane protein involved lymphocytes from a patient with Graves disease without ophthalmopathy and occurred with both Tg and TgA. The single exception for kidney membrane protein occurred with lymphocytes from a patient with Graves ophthalmopathy, and lysis was observed with both Tg and TgA. We are unable to explain either of these exceptions.

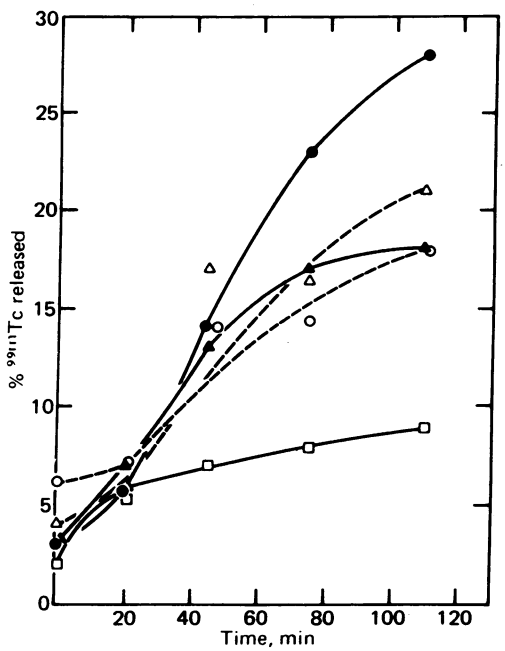

FIG. 3. High-affinity E-rosette forming (T) cells and low-affinity E-rosette forming (K) cells were obtained from a patient with Graves ophthalmopathy. The separated K (—) and T (---) cells were incubated with human EMP-vesicles as described in the legend to Fig. 1, in the presence of Tg (▲, △) or TgA (●, ○). Vesicles alone (□) were used to measure "control" release of radioactivity in the absence of lymphocytes, Tg, or TgA. A repeat experiment with lymphocytes from the same subject gave similar results.

DISCUSSION

The results of the present study indicate that cell-mediated lysis of artificial lipid vesicles can be achieved within a narrowly specified set of conditions. That cell-mediated lysis has been achieved with EMP-vesicles is indicated by the fact that lymphocytes are required, and that an immune complex (TgA) is required for lysis by lymphocytes from normal subjects, while antigen only (Tg) suffices for lysis by lymphocytes harvested from patients with Graves ophthalmopathy. The latter are patients whose sera frequently contain anti-Tg (8) and whose peripheral blood leukocytes respond *in vitro* with a characteristic inhibition of migration when exposed to orbital muscle homogenate or Tg (4–6). However, it is significant that the presence of lymphocytes and antigen or antigen–antibody complex alone is not sufficient to induce lysis: our data and that of our colleagues (T. Lewis and H. McConnell, personal communication) indicate that vesicles containing phospholipids and cholesterol alone are not subject to cell-mediated lysis. It is first necessary to insert a protein (solubilized human or bovine EMP) that has some affinity for Tg. For this purpose we prefer the human rather than bovine source, because occasional batches of bovine EMP have lacked binding affinity for labeled Tg when tested *in vitro* in the presence of normal human IgG. Such batches will not yield satisfactory EMP-vesicles for lysis experiments.

Although binding of Tg to EMP-vesicles is tested in hypotonic solution and requires the presence of normal IgG, vesicle lysis occurs at 37°C in isotonic solution at pH 7.4 and normal IgG is not required. We know of no other cell membrane protein that will substitute for EMP in our system. Notably ineffective were solubilized membrane proteins of human origin made from gluteal or cheek skeletal muscle, liver, spleen, and adrenal gland. With single unexplained exceptions in one run, human thyroid and kidney membranes were also ineffective in yielding a satisfactory protein. Each of the above membrane preparations has been checked by electron microscopy (unpublished observations).

How does one account for the apparently unique qualitities of EMP? Three main possibilities occur to us: (*i*) EMP contains an ingredient not shared by other muscle which serves as attachment sites for Tg or TgA, (*ii*) EMP contains an antigen to which some lymphocytes (e.g., those from patients with Graves ophthalmopathy) are sensitized, and (*iii*) EMP, but not other membrane proteins, when inserted into the vesicle wall, has some structural characteristic that renders the vesicle wall breakable. None of these possibilities is mutually exclusive. The second possibility is suggested by our observation that vesicle lysis does *not* occur when vesicular EMP is totally replaced by Tg, even when lymphocytes (obtained from patients with Graves ophthalmopathy) that lysed EMP-vesicles in the presence of Tg were used. If the third possibility were true, then it should be possible to induce cell-mediated vesicle lysis with some other specific antigen and antibody combination.

Eye muscles are unique in a number of respects. They do not form bundles, they are short, and they have a very high nerve:muscle fiber ratio compared to other skeletal muscle (21). They also differ significantly from other striated muscle on examination by light microscopy, electron microscopy, and enzyme histochemistry (22, 23). We do not know which component of the eye muscle protein preparation is crucial to induction of cell-mediated lysis.

We believe there is considerable clinical relevance of our observations to the pathogenesis and treatment of patients with Graves disease. Although the numbers tested are few, the fact that lymphocytes from each patient with Graves ophthalmopathy induced vesicle lysis both with Tg and TgA, while lymphocytes from patients with Graves disease without ophthalmopathy lysed only with TgA, suggests that sensitization to Tg in the former group is clinically relevant. Thus, eye muscle damage may occur either by T-cell sensitization to Tg (attached to the eye muscle cell) or by K-cell attack initiated by recognition of TgA (attached to the eye muscle cell). We do not know how or when patients with Graves ophthalmopathy acquire their Tg-sensitized T cells, the duration of sensitization, or the consequence of a loss of sensitization, or how safely to desensitize. Prior knowledge of T-cell sensitization to Tg in Graves disease patients before treatment might well affect treatment decisions. For example, one might choose to avoid thyroid ablative therapy for such patients, since severe ophthalmopathy has been observed to follow such treatment (3, 24), or one might consider the prophylactic use of systemic corticosteroids soon after [^{131}I]iodide therapy, when release of Tg from the thyroid is maximal. A successful prophylactic immunosuppressive regimen by use of azathioprine has in fact been claimed (25).

It will be especially interesting to perform the test with lymphocytes herein described to study those euthryoid patients with ophthalmopathy who have no previous history of thyroid disease or laboratory evidence of thyroid dysfunction or autoimmunity (26) in order to determine whether these patients nonetheless share the described immunologic feature observed in those having classical Graves ophthalmopathy. If not, it would seem reasonable to search for a different etiology and to assign a separate nomenclature.

To date we have observed one healthy control subject whose lymphocytes lyse EMP-vesicles in the presence of Tg. Neither she nor any member of her family is known to have had thyroid or eye diseases. Thus, it is possible to have Tg-sensitized lymphocytes without noticeable harmful consequences, presumably so long as thyroid hyperfunction and excessive Tg secretion are not present.

The use of vesicles incorporating eye muscle cell protein offers opportunities to study the mechanism of cell-mediated killing and the critical parameters for such killing and may afford a technique for the study of cell-mediated immunity involving other organs or tissues.

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