Trypsin Esterase Binding and Its Relation to the Radiation-Leukemia Protection (RLP) Factor[1] (34672)

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The RLP (radiation-leukemia protection) activity of sheep spleen extracts is sharply eluted from the first Sephadex G-200 peak with the proteins of higher molecular weight ("19S peak"), whereas in serum the activity is more diffuse and considerable amounts appear in the lighter fractions (1). Berenblum and his co-workers have suggested that RLP is an α_2-macroglobulin (α_2M) (2). As α_2M is readily detectable by its ability to bind trypsin causing protection of the esterase activity of the enzyme from inactivation by trypsin inhibitor (3), we have determined the distribution of the trypsin-esterase binding activity (TEBA) of serum and spleen extracts. The activity follows the RLP elution pattern for spleen but not for serum. Furthermore, the TEBA of serum and spleen exhibit differences in stability to heat and pH changes. In addition to the TEBA of serum and spleen, another characteristic property of α_2-macroglobulin, the trypsin inhibiting capacity (TIC) has also been investigated.

Materials and Methods. The preparation of sheep spleen extracts (40% homogenates in phosphate-buffered saline, pH 7.4, centrifuged 1 hr at 25,000 rpm in a Spinco rotor No. 30 in the cold) and fractionation of extracts and serum have been described (1). Fractionations were performed on a column of Sephadex G-200, equilibrated with phosphate-buffered saline. Both TEBA and TIC were determined by a modification of the methods of Ganrot (3, 4). The determinations were performed on 5-μl samples and the change in absorbancy at 410 mμ was recorded for at least 10 min in a Gilford spectrophotometer at 25°. Immunoelectrophoresis was performed in a Gelman apparatus in 1% agar at pH 8.6 in 0.1 *M* buffer.

Results. The fractionation on Sephadex of the substances precipitable upon 40% saturation of spleen extract with ammonium sulfate differs from that of the comparable serum preparation. A greater proportion of the total protein of the spleen preparation appears in the 19S peak and the TEBA of spleen appears 4 fractions earlier (Fig. 1). The TEBA of both spleen and serum follows the 19S peaks fairly closely, although the elution is sharper from serum. A considerable portion of the TEBA, especially of serum, fails to precipitate at 40% saturation with ammonium sulfate and appears in the 40–60% precipitate. This is rather sharply eluted with the 19S material (Fig. 1D).

The relation of the TEBA to the α_2M content of the fractions is evident on immunoelectrophoresis (Fig. 2). A precipitin line in the α_2M position is found only in fractions 1 and 2.

The TEBA of serum and spleen differ in stability to heat and pH changes. The activity of spleen is more sensitive to heating at 60° (Table I). Under dialyzing conditions, the serum activity is stable at pH 4.5, whereas the spleen TEBA is almost inactivated. At pH 9.5, both spleen and serum retain 65–72% of their activity. The activity of spleen exposed to pH 4.5 can be partially

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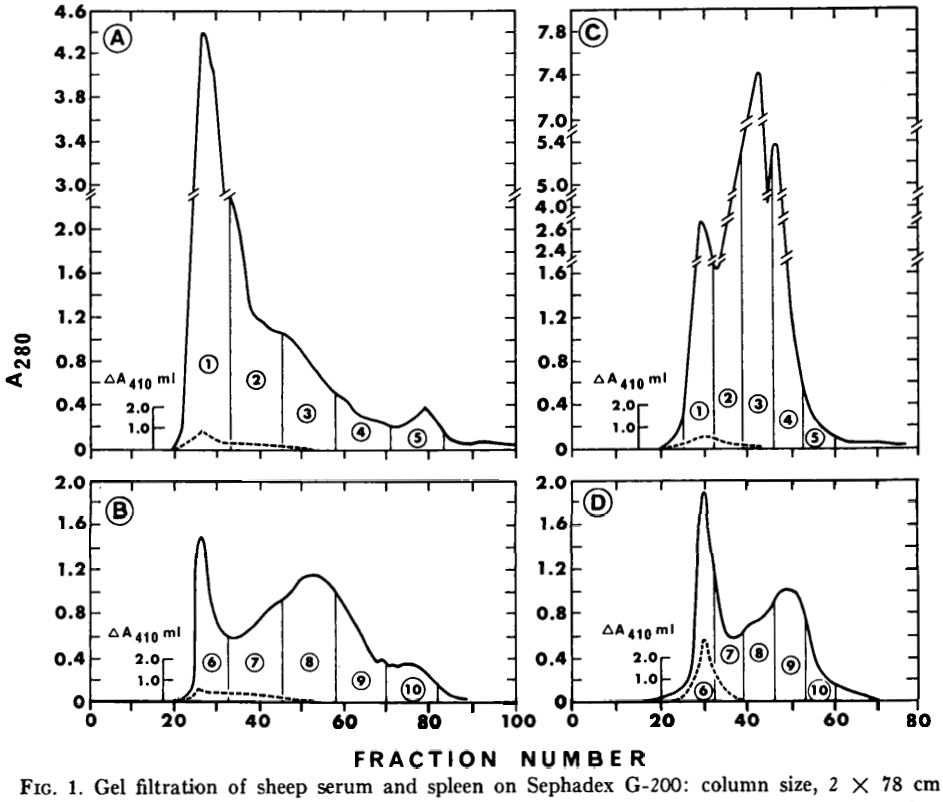

FIG. 1. Gel filtration of sheep serum and spleen on Sephadex G-200: column size, 2 × 78 cm; sample size, 2 ml; flow rate, 7.2 ml/hr. (A) sheep spleen extract, 40% saturated ammonium sulfate precipitate; (B) sheep spleen extract, 40–60% saturated ammonium sulfate precipitate; (C) sheep serum, 40% saturated ammonium sulfate precipitate; (D) sheep serum, 40–60% saturated ammonium sulfate precipitate; (—), the absorption at 280 mμ; and (---), the TEBA activity.

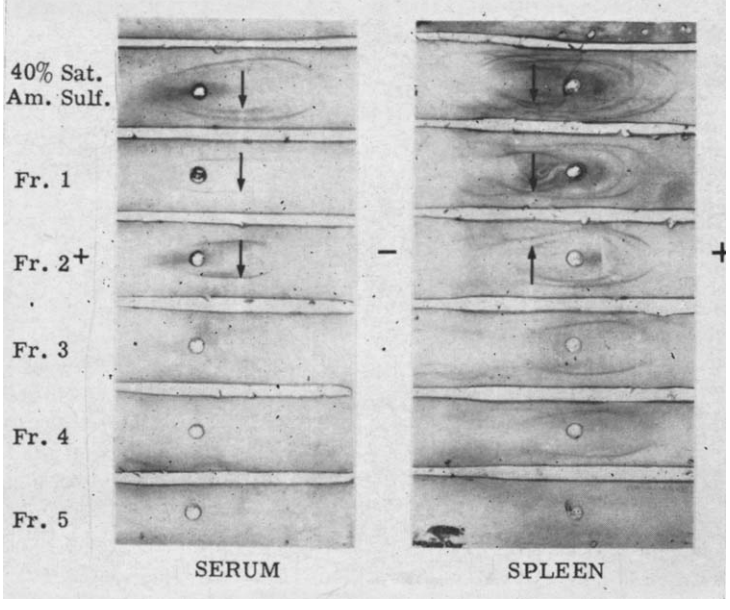

FIG. 2. Immunoelectrophoresis of fractions eluted from Sephadex G-200 (Fig. 1A and C). The trough contains rabbit antisheep serum. Arrows indicate the α_2M precipitin line.

TABLE I. The Effect of Heating at 60° on the Trypsin Esterase Binding Activity of Sheep Serum and Spleen.

Time at 60° (min)	% Untreated activity	
	Serum	Spleen
0	100	100
5	103.6	30
15	81.3	19
30	80.6	0
60	64.5	0

restored by dialysis against pH 9.5 buffer (Table II).

The TIC of sheep serum is present in most of the Sephadex G-200 fractions. The TIC of spleen is confined to the 19S material and also corresponds to the TEBA of that peak. After the fractions obtained by gel filtration of spleen extract were heated at 60° for 10 min at pH 7.4, neither the TEBA nor the TIC could be detected. Heating under the same conditions had no effect on serum TEBA and caused but little inactivation of serum TIC in the later fractions (data not shown).

After exposure of mice to 300 R, the TEBA of their serum decreases and remains depressed for several days. Injection of sheep serum, but not saline or albumin, restores the TEBA (Fig. 3). Similar results were obtained with C57Bl/6 and Swiss mice. The

TABLE II. α_2M Trypsin Esterase Binding Activity of Sheep Spleen Extract and Serum.

Change of pH was accomplished by dialysis at 4° for 8–24 hr.

Treatment (pH)	% Untreated activity	
	Serum	Spleen
9.5	72.8	65.0
4.5	96.6	9.1
9.5→4.5	75.2	10.5
4.5→9.5	96.6	37.7

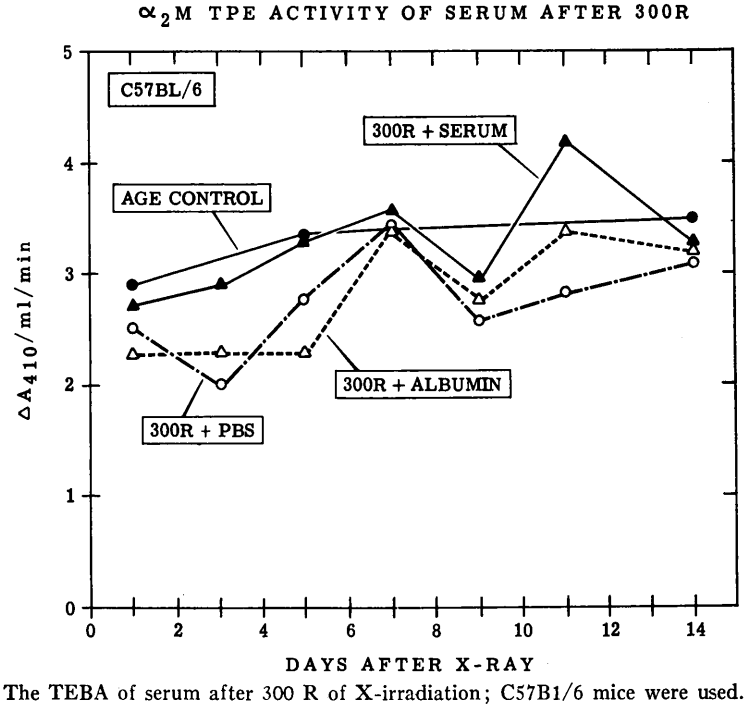

FIG. 3. The TEBA of serum after 300 R of X-irradiation; C57Bl/6 mice were used. Each point on the graph is the average value for 6 determinations: (●) age control, no treatment was given; (▲) mice received 300 R of X-ray plus injections of 0.25 ml of serum right after the irradiation. (○), mice received 300 R of X-ray plus injections of 0.25 ml of PBS right after the irradiation; (△), mice received 300 R of X-ray plus injections of 0.25 ml 1% albumin in PBS immediately after irradiation.

differences were examined in an analysis of variance and found to be significant at the 5% level. The localization of this activity was not attempted.

Discussion. The elution from Sephadex of the α_2M of sheep serum, as estimated by its trypsin esterase binding activity, is similar to that described for α_2M from human serum (4). However, the sheep serum TEBA differs from that of human serum in its stability to heat inactivation. On the other hand, sheep spleen TEBA is thermolabile and acts more like the human α_2M described by Ganrot (4). Since the TEBA of spleen appears 4 fractions earlier than that of serum on elution from Sephadex and there is a complete lack of any other antitrypsin activity in its fractions, it is not likely that the activity of the spleen TEBA is attributable merely to contamination by serum.

The quanternary structure of α_2M varies with pH. The 17.5S form of α_2M predominates between pH 3 and 7.5, whereas at pH 9 most of the substance is in the 12S form (5). Unless human and sheep α_2M differ vastly, it is not likely that dissociation alone plays a major role in the activity changes seen in Table II, as fresh spleen extract (pH 6.5) is active in binding trypsin esterase, although activity is lost when the pH is lowered to 4.5.

The relationship of the RLP activity of spleen and serum to α_2M remains obscure. Both α_2M and RLP are rather sharply eluted with the first peak of protein in sheep spleen extracts. However, RLP of serum is rather diffusely distributed (1), whereas the α_2M is limited to the early fractions (Fig. 1). The TEBA of mouse serum falls after X-irradiation and is restored by injection of sheep serum which exhibits RLP activity, but not albumin or saline, which do not protect against radiation-induced leukemia (Fig. 3). Exposure to X-rays is known to interfere with macroglobulin synthesis (6), but it also causes many changes in serum enzyme levels of questionable specificity. These changes can be reversed by injection of serum, but not by albumin (7).

The differences in TEBA of spleen and serum—in precipitation by ammonium sulfate, elution from Sephadex, and heat and PH stability—and the differences in distribution of spleen and serum RLP activity lead us to believe that RLP may exist as a complex with α_2M. The binding between α_2M and RLP is tight in spleen, causing both to elute sharply near the void volume. The activities are probably readily dissociated in serum, and the relatively small RLP molecule may then appear free or partially associated with various other proteins.

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1. Clewell, D. B., Hubbard, J. D., Yu, P.-L., Yip, L. C., and Hodes, M. E., Cancer Res. **29**, 1333 (1969).

2. Berenblum, I., Burger, M., and Knyszynski, A., Nature (London) **217**, 857 (1968).

3. Ganrot, P. O., Clin. Chim. Acta **14**, 493 (1966).

4. Ganrot, P. O., Clin. Chim. Acta **13**, 597 (1966).

5. Gentow, C., C. R. Acad. Sci. **266**, 2358 (1968).

6. Berlin, B. S., J. Immunol. **93**, 315 (1964).

7. Yip, L. C., Blair, N., Yu, P.-L., and Hodes, M. E., Isr. J. Med. Sci. 4, 1188 (1968).

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