Ultrastructure of Lymphocyte-Mediated Cytotoxicity. II. Interaction Between Human Lymphocytes and Antibody-Coated Chicken Erythrocytes

In a previous study, the ultrastructural appearance of the interaction between normal human phytohemagglutinin (PHA) – stimulated lymphocytes and chicken erythrocytes (ChRBC) was investigated[1]. Interdigitations occurring between effector and target cells were considered the morphological counterpart of the cytotoxic effect of lymphocytes, since they appeared with, and followed the same time course of radiochromium (^{51}Cr) release from ChRBC. Close contact between lymphocytes and ChRBC[2] only was considered non-significant, since it occurred frequently also in the absence of cytotoxicity. In this report we present the results of a study, performed by the same techniques, of the interaction between normal human lymphocytes and chicken erythrocytes coated with specific antibody (Ab-ChRBC) at the ultrastructural level.

Materials and methods. The preparation of lymphocytes obtained from healthy volunteers and of target cells and the radiochromium labeling of the latter have already been described[1]. For the cytotoxicity assay, 0.5 ml of lymphocytes suspension were mixed with 0.5 ml of ChRBC suspension in test tubes, to each of which 0.5 ml of rabbit anti-ChRBC antiserum were added at optimal concentration (10^{-4} dilution). The tubes were incubated in the same conditions as our previous experiment[1] for 10, 30, 45 min and for 1, 2, 4, 8, 18, 20, 24 h. The cytotoxic effect of lymphocytes was evaluated by measuring

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Kinetics of human normal lymphocyte cytotoxicity on radiochromium-labelled Ab-ChRBC and of lymphocytes-Ab-ChRBC interdigitation

Incubation time	Incubation mixtures[a]			
	Lymph. + Ab-ChRBC		Lymph. + ChRBC + RNS[b]	
	^{51}Cr release (%)[c]	Interdigitation (%)[d]	^{51}Cr release (%)[c]	Interdigitation (%)[d]
0	1.0	0	0.5	—
10 min	3.0	25	0.5	—
30 min	4.0	28	1.0	—
45 min	6.5	28	1.2	—
1 h	8.0	32	1.2	—
2 h	17.0	32	1.5	—
4 h	35.6	—	1.5	—
8 h	65.0	—	2.0	—
18 h	71.5	—	2.0	—
20 h	80.2	—	2.0	—
24 h	84.0	—	2.0	—

[a] Lymphocytes/target cells ratio 25/1. [b] Rabbit normal serum. [c] Values were corrected for the spontaneous radiochromium release from ChRBC incubated without lymphocytes. [d] The percentage (%) was calculated on 200 ChRBC.

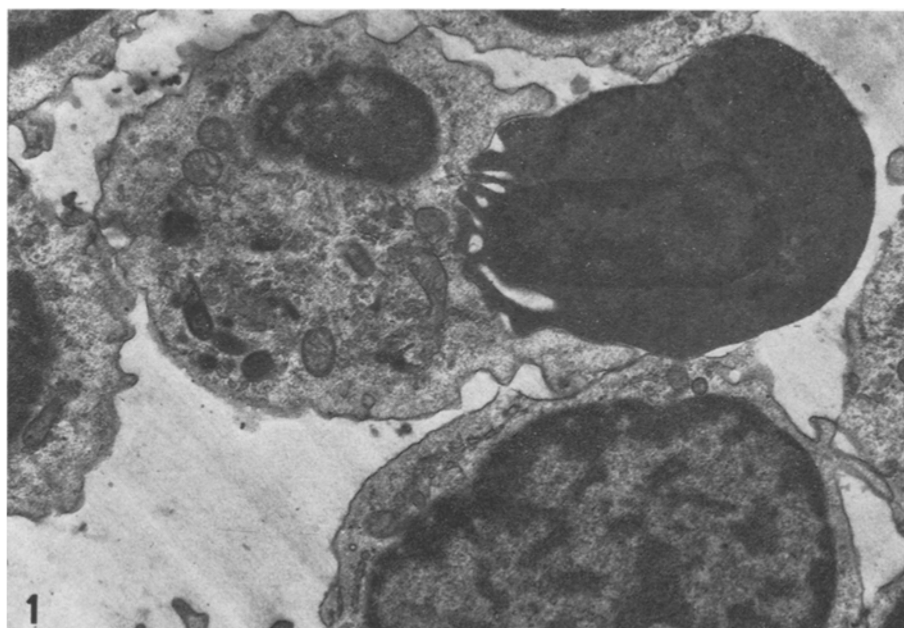

Fig. 1. Lymphocytes-Ab-ChRBC interaction, 10 min incubation. An interdigitation between effector and target cell is visible. ×14,200.

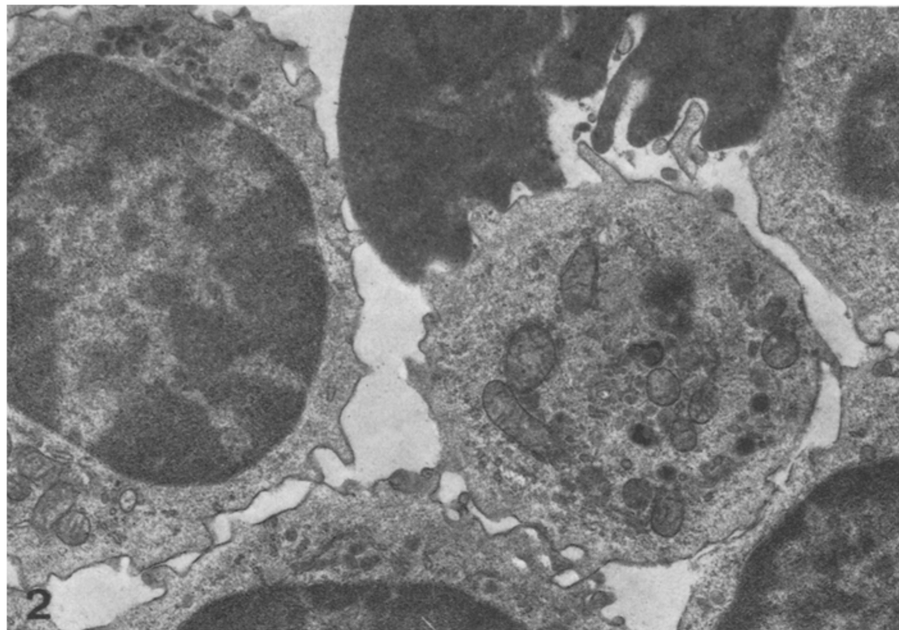

Fig. 2. Lymphocytes-Ab-ChRBC interaction, 30 min incubation. 1 Lymphocyte interdigitates with 2 Ab-ChBRC. × 14,900.

the radiochromium release from Ab-ChRBC at the different incubation times. As a negative control, lymphocytes were incubated with radiochromium-labelled ChRBC with addition of decomplemented serum obtained from non-immunized rabbits. At each incubation time, aliquots of the samples were processed for electron microscopy[1]. An identical set of experiments were performed using non-labelled ChRBC in order to exclude any possible artifactual effect of radiochromium on the ultrastructural appearance of Ab-ChRBC.

Results. Quantitation of cytotoxicity. Radiochromium release from Ab-ChRBC is shown in the Table. The cytotoxic effect rapidly increased between 20 min and 6 h and then slowly to 84% at 24 h. In control experiments, radiochromium release never exceeded 2%. Ultrastructural study. In the samples obtained from the cytotoxicity assays, an incidence of interdigitations between effector and target cells of about 20% was already present after only 10 min of incubation (Table and Figure 1). By 4 h, interdigitations were no longer visible. In several instances, a single lymphocyte interdigitated with more than one Ab-ChRBC (Figure 2). Only rarely 1 Ab-ChRBC interdigitated with 2 lymphocytes (Figure 3). No evident morphological differences were observed between interdigitating and non-interdigitating lymphocytes. In control experiments, no interdigitations were observed. No differences were observed in the incidence of interdigitations by use of radiochromium-labelled or unlabelled Ab-ChRBC.

Discussion. The results of this study are in agreement with those obtained in PHA-induced cytotoxicity[1] as far as the morphological phenomena of interdigitation are

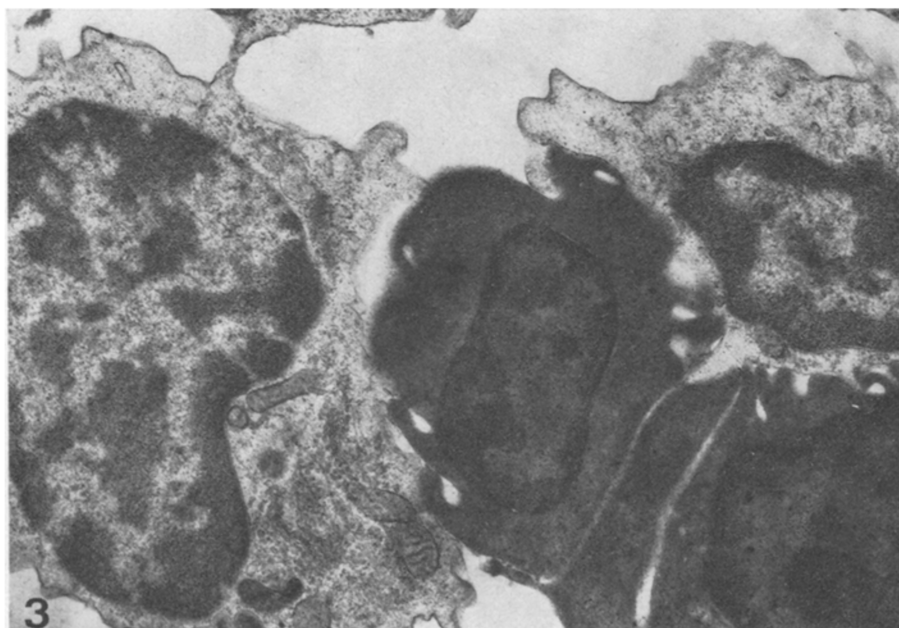

Fig. 3. Lymphocytes-Ab-ChRBC interaction, 1 h incubation. 1 Lymphocyte interdigitates with several Ab-ChRBC. One of the latter interdigitates also with a second lymphocyte. × 15,600.

concerned. However, the correlation between appearance and percentage of radiochromium release and of interdigitations is much stricter in PHA-induced than in antibody-mediated cytotoxicity. The different kinetics of the two cytotoxic phenomena may be explained by the fact that the effector cells are not the same in the two systems. In PHA-induced cytotoxicity, the effector cells are considered to be T cells, whereas in antibody-mediated cytotoxicity the effector cells, which have receptors for the Fc fragment of immunoglobulins, may belong to a subpopulation of B cells lacking surface immunoglobulins, or to an entirely different population of lymphocytes. The former type of cytotoxicity implies cooperation between several cell types which is probably not necessary in the latter[3–6]. These functional differences are not correlated with specific ultrastructural characteristics which might allow a distinction between these different cell types on morphological grounds. It seems likely that in PHA-induced cytotoxicity, the activation of effector cells and their contact with and consequent damage to target cells is slower and more progressive, presumably because of the more complex mechanisms of cell cooperation mentioned above. The interdigitation phenomenon appears identical in the two types of cytotoxicity. The relatively frequent occurrence of interdigitations between a single lymphocyte and several Ab-ChRBC is in agreement with the observation that presumably each effector cell has the capacity of destroying several target cells[6] and, according to our findings, also at the same time.

The observation of interdigitations, also in those cytotoxicity assays in which Ab-ChRBC had not been labelled with radiochromium, seems to exclude any relevance of the tracer in the induction of the phenomenon. Once established, as it is in our opinion, that interdigitation is the morphological evidence of the cytotoxic effect of lymphocytes on target cells, the intimate mechanisms by which this phenomenon occurs remain to be elucidated. Studies are now in progress to investigate the changes occurring in the electric charges at the surface of cells during the phenomenon in order to establish whether or not they may play a major role in it.

Summary. In the course of antibody-mediated lymphocyte cytotoxicity, ultrastructural studies show interaction between effector and target cells characterized by interdigitations. The significance of the observation is discussed.

L. Fontana, G. De Sanctis, G. Pecci and G. Tonietti[7]

I. Patologia Medica, University of Rome and II. Clinica Medica, University of Rome, Policlinico Umberto 1, I–00161 Roma (Italy),
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Production of Antibodies to Amanitins as the Basis for Their Radioimmunoassay

Amanitins[1,2] are the main toxins of the toadstools *Amanita phalloides* (Death Cap) and *Amanita verna* (Destroying Angel) which are responsible for some hundreds of poisonings, with many deaths, every year[3]. There has been no method, until now, for measuring the concentrations of these toxins in biological fluids. We describe here a radioimmunoassay which allows detection of as little as 0.5 ng of amanitins per ml of serum.

Since the first attempt of Calmette in 1897, several efforts were made in the past to obtain an antiserum to the toxins of *Amanita phalloides* by injecting animals with extracts of the toadstool[4]. The low molecular weight of the toxins (about 900) accounts for the failure of these attempts[5]. More recently β-amanitin was made antigenic by conjugating it to proteins[6–8]. The conjugates proved to be very toxic for those cells which display a high protein uptake, such as liver sinusoidal cells[9], proximal tubule cells of kidney[10], and macrophages[11]. As a consequence of this toxicity they could not be administered to rabbits and mice in immunogenic doses[7].

In the experiments reported here, we succeeded in obtaining antibodies against amanitins by injecting immunogenic doses of an amanitin-albumin conjugate into the rat, which is several times more resistant to amanitins[12], as well as to amanitin-protein conjugates[13], than rabbits and mice.

For the preparation of the conjugate, β-amanitin (16 mg) was allowed to react with 30 mg of rabbit serum albumin (RSA) and 13 mg of 1 cyclohexyl-3-(2-morpholinyl-(4)ethyl)carbodiimide (Morpho-CDI) in 1.5 ml of water at 22 °C for 24 h. The conjugate (amanitin-RSA) was separated from free β-amanitin and from unreacted Morpho-CDI by gel filtration on a 1.2×100 cm column of Sephadex G-75 equilibrated and eluted with 0.9% NaCl solution. The molar ratio of amanitin to albumin in amanitin-RSA, calculated according to Derenzini et al.[9], was found to be 1.4. The conjugate was brought to a protein concentration of 4 mg/ml by vacuum dialysis against 0.9% NaCl and was emulsified with an equal volume of complete Freund's adjuvant.

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