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Granulocytes as Effectors in Cell-mediated Cytotoxicity of Adherent Target Cells[1]

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SUMMARY

In the microassay for cell-mediated immunity, detachment of adherent target cells from the wells occurs to an even greater extent when tested with granulocytes than when tested with lymphocytes. Intact cells are not necessary since the sonic extract from granulocytes causes the same effect. The reaction by granulocytes appears to be mediated by enzymes, and is inhibited by the presence in the wells of hyaluronic acid. Moreover, it is limited to the detachment of adherent target cells, since neither intact nor sonically disrupted granulocytes exhibit cytotoxic activity in the chromium release assay. The detachment of target cells is also inhibited by heparin which may be used to specifically nullify the effect of granulocytes contaminating lymphocyte suspensions.

INTRODUCTION

Detachment of adherent target cells from surfaces as a criterion for cell death has been used in cell-mediated cytotoxic studies on monolayers and in microtest wells. The strength of this reaction is evaluated from counts of surviving target cells or isotopic labeling from these cells. The specificity of this reaction in testing human cancer patients has been claimed to be related to the "histological" type of tumor (1-3, 5-8, 10, 12-15, 17-19). However, in some studies, such claims of specificity were made when lymphocyte suspensions with up to 30 to 40% granulocytes (8, 12), or more were used (5). Lundgren *et al.* (16) have previously shown, and we have confirmed (22), that granulocytes cause strong nonspecific target cell detachment that would strongly interfere with the detection of any specificity. In the present study, the effect of granulocytes, their mode of action on the detachment of target cells, and their influence on the detection of specificities were reexamined.

MATERIALS AND METHODS

Isolation of Granulocytes. Peripheral blood was collected in 10- to 15-ml heparinized vacutainer tubes and centrifuged (International Model UV centrifuge; Needham Heights, Mass.) at 700 × *g* for 10 min. The buffy coat was carefully aspirated and mixed with an equal volume of culture media used for growing target cells. The suspension was layered over a 1.5-ml Ficoll-Hypaque solution (4) in a 5-ml tube and centrifuged again for 10 min at 600 × *g*. Lymphocytes were found mostly in the interface layer. For preparation of granulocytes, the upper layer of the pellet was retrieved, and contaminating erythrocytes were removed by agglutination with anti-A, anti-B, or anti-H, and light centrifugation (2 sec at 1000 × *g* in a Fisher centrifuge). The supernate was transferred to a 2nd Fisher tube containing 0.3 ml Ficoll-Hypaque and recentrifuged for 1 min at 1000 × *g* (Fisher centrifuge). The pellet was washed twice with media and used if the suspension contained more than 90% granulocytes.

Sonic Disruption. Cells to be treated were adjusted to 10 × 10^6/ml and treated sonically for 1 min on high adjustment of the ultrasonic generator (Electrosonic Systems, Los Angeles, Calif.). The sonic extract was centrifuged to remove large debris, and the supernatant was tested.

Target Cells. Target cells used in the study were the cultured human tumor lines and fibroblasts listed in Table 1. The cells were cultured and tested in minimum essential medium supplemented with 20% fetal bovine serum and penicillin, streptomycin, gentamicin, and amphotericin B.

The Microassay for Cell-mediated Immunity. The assay system for testing lymphocytes has been described previously (20-23). For this study, purified and sonically disrupted granulocytes were used as effectors. Briefly, 4 dilutions of effector cells from 50,000 to 6,000 were reacted with target cells in Falcon 3034 microtest plate I (Falcon Plastics, Oxnard, Calif.). After 2 days of incubation, surviving cells were fixed, stained, and counted electronically. For inhibition of the granulocyte effects, 5 µl media, 5 µl granulocytes, and 5 µl media with heparin, 30 units/ml, were added to give a final heparin concentration of 10 units/ml. In some experiments, effector cells equivalent to the highest concentrations of intact cells used were sonically disrupted and tested.

Titration of Enzymes. Three enzymes were tested for their ability to cause detachment of cultured target cells. These were hyaluronidase from bovine testes, B grade (Calbiochem, La Jolla, Calif.), lysozyme (received through the kind courtesy of Dr. E. Sercarz, University of California, Los Angeles, Calif.), and β-glucuronidase from B-grade bovine liver (Calbiochem). Each enzyme was titrated at 1:2 consecutive dilutions from an original concentration of 80 mg/ml, and 2 µl of each titration were added to each well containing 10 µl media and adherent cells. For possible inhibition of the

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Table 1
Human tumor lines and fibroblasts used as target cells

Identification no.	Other designations	Cancer derivation of target cell cultures	Received from
124		Normal fibroblast	
125	Hep-2	Larynx	
133		Osteosarcoma	E. T. Bloom
370	BT20	Breast	J. L. McCoy, R. B. Herberman
372	Me180	Cervix	E. T. Bloom
373	HT29	Colon	J. C. McCoy, R. B. Herberman
462	G5	Breast	E. J. Plata
463	G11	Breast	E. J. Plata
470		Melanoma	S. H. Golub
471		Sarcoma	S. H. Golub
496	AlAb	Breast	E. T. Bloom
497		Leiomyosarcoma	E. T. Bloom
499		Basal cell	
516		Tongue	
546	Lev	Melanoma	J. L. McCoy, R. B. Herberman
548		Breast	
696	2T	Sarcoma	H. Oettgen, M. Bean
697	T24	Bladder	H. Oettgen, M. Bean
719		Normal fibroblast	
763		Larynx	
805	HT39	Breast	E. J. Plata
917	BeRo	Melanoma	B. Mukherji
1042		Sarcoma	J. Heppner
1210		Lip	P. Ward
1233		Sarcoma	

enzymes, 5 μl of culture media containing heparin, 30 units/ml, were added for a final concentration of approximately 9 units/ml. The plates were then incubated and terminated as in other tests.

Analysis. The effect of granulocytes on target cells was measured by comparing the number of surviving target cells with the number of cells in the medium control. The percentage of reduction was calculated for the 4 dilutions of granulocytes or the sonic extract and averaged to give the MRT score (24).

Chromium Release Assay. Target cells selected for low background were harvested from tissue culture and labeled with 50 to 100 μCi ^{51}Cr (Amersham/Searle Corp., Arlington Heights, Ill.) at 37° for 1 hr. The cells were washed 3 times in media before being used as target cells. All tests were conducted in triplicate in gas-sterilized Beckman centrifuge tubes. A test consisted of target cells with medium used only to measure spontaneous release (minimum), target cells in distilled water (maximum), and reactions between 100,000 effector cells, intact or sonically disrupted, and 2000 target cells in a total volume of 0.2 ml. After 16 hr of incubation in a CO_2 humidified incubator, the tubes were centrifuged, and 0.1 ml supernatant was recovered. This supernatant (*A*) and the original tube (*B*) with cells and remaining media were counted, and the release of ^{51}Cr was calculated from the mean for each triplicate as follows: ^{51}Cr release = $2A/A + B$.

$$\% \, ^{51}\text{Cr release} = \frac{\text{Test release} - \text{minimum release}}{\text{Maximum release} - \text{minimum release}} \times 100$$

RESULTS

While considerable variability exists in the ability of lymphocyte suspensions from different individuals to reduce the number of surviving target cells (24), purified granulocytes are consistently strong in this respect. Table 2 shows the effect of 8 granulocyte suspensions tested on 4 different target cells. The effect was usually stronger than for a corresponding number of lymphocytes and was correlated with the number of granulocytes applied.

To determine whether this reaction possessed any specificity, 8 granulocyte suspensions were tested in 2 series against 7 different target lines (Table 3). As specific reactions are difficult to demonstrate in lymphocyte-mediated tests, the absence of specificity with granulocytes causes similar problems. Most targets were highly sensitive to granulocytes, exhibiting high MRT scores over a limited range, so that precise differentiation according to the strength of reaction was not possible. In others, a greater spread in the scores was observed, with general agreement for the strong and weak reactors. No indications of specific reactivity toward a target were observed, and the differences in the sensitivity of a target culture to granulocyte reactions influenced the results even more than did the effector granulocytes tested.

Intact granulocytes are not necessary, since equivalent concentrations of sonically treated cells also reduced target cell numbers. Granulocytes were sonically treated until no intact cells could be observed microscopically, and the mixture was tested at 4 dilutions. The results were slightly

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Table 2

Effect of purified granulocytes on cultured adherent target cells

Granulocyte no.	Target no.	Surviving target cells after test with granulocytes					MRT score
		50,000	25,000	12,500	6,250	0	
52647	499	9	7	9	29	60	70
53293	496	1	3	17	28	90	85
53460	516	5	19	15	68	59	60
53527	470	21	32	45	61	115	64
54008	696	1	4	7	10	57	89
54402	373	13	22	19	20	109	82
54330	719	50	78	82	114	106	27
		14 ± 7	24 ± 10	28 ± 10	47 ± 14	85 ± 10	68 ± 8

Table 3

Effect of granulocyte samples on various target cells

Granulocyte no.	MRT score on targets							Mean ± S.E.
1st series	372	462	548	696	697	1042	1210	
77305	33	72	86	79	92	54	99	74 ± 9
77317	13	67	82	92	89	51	98	70 ± 11
77324	35	94	83	82	89	80	98	80 ± 8
77325	28	86	81	89	91	51	97	75 ± 10
2nd series								
77264	79	86	87	56	93	76	95	82 ± 5
77270	71	91	84	77	82	75	96	82 ± 4
77271	37	84	74	52	88	77	97	73 ± 8
77269	46	82	73	54	88	58	28	61 ± 8
Mean ± S.E.	43 ± 8	83 ± 3	82 ± 2	73 ± 6	89 ± 1	65 ± 5	89 ± 9	

Table 4

Effect of sonically disrupted granulocytes on cultured adherent target cells

Sonic extract no.	Target no.	No. of surviving target cells after reaction with granulocytes					MRT score
		50,000	25,000	12,500	6,250	Medium	
77454	372	25	198	273	428	491	52
	462	63	75	98	350	449	67
77456	548	58	72	119	121	121	36
	697	28	59	116	130	194	64
77309	463	64	175	298	407	541	55
	917	305	284	318	397	436	24
Mean ± S.E.		91 ± 44	144 ± 37	204 ± 42	306 ± 58	372 ± 70	507 ± 7

weaker than for intact cells but were similar in activity, with survival of target cells correlated to the dilution of the sonic extract (Table 4).

The question of whether the same cause produced the effect by intact and sonically disrupted granulocytes was tested in 2 ways. Granulocytes and sonic extracts from the same person were tested on different targets (Chart 1) and, also, effectors from different people were tested on the same target (Chart 2). The 1st combinations indicated that the sensitivity of the target cells to reactions with intact cells was correlated to the sensitivity of the target to the sonic extract with a coefficient of 0.7 and a probability of 0.026. By the 2nd method, the strength of reaction of the granulocyte and the corresponding sonic extract was correlated with a coefficient of 0.89 and a probability of less than 0.0001. We conclude that the same reactions that cause reduction of target cells from granulocytes occur for their sonic extract.

The detachment of target cells by granulocytes may be inhibited by the addition of heparin. Table 5 shows the number of target cells surviving after reaction with granulocytes in tests with varying concentrations of heparin. Inhibition of the granulocyte-mediated target cell loss correlated with increasing amounts of heparin, and most target cell reduction was neutralized by 10 units of heparin per ml. Nearly identical results were obtained with sonically

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treated granulocytes, confirming the conclusion that the same underlying phenomenon was the cause of these effects.

That heparin was specifically responsible for the inhibition of granulocyte- and sonically treated granulocyte-mediated target cell release was shown by reversing the effect of heparin with protamine sulfate (Table 6) (11). Inhibition of target cell release by heparin, 10 units/ml, was effectively counteracted by protamine sulfate, 10 mg/ml, so that target cell reduction approximated results in the absence of heparin. Protamine by itself had no effect on the result.

The effect by sonically treated granulocytes and the inhibition of this effect by heparin suggested that enzymes and possibly lysosomal enzymes were responsible for the detachment of target cells. Table 7 shows the effect of titration of 3 enzymes, hyaluronidase, lysozyme, and β-glucuronidase. Only hyaluronidase caused target cell release comparable to the effect of sonically treated granulocytes. Whether hyaluronidase from granulocytes was responsible for the effect could not be definitely determined, since the enzymes available were derived from sources other than human granulocytes, and heparin at concentrations that inhibited the granulocyte effect showed little or no inhibition of the enzyme. However, further indications that hyaluronidase or similar polysaccharidases are the cause of target reduction developed from studies in which varying amounts of hyaluronic acid were added to the test with sonic extract from granulocytes. Table 8 shows the results in which average MRT scores from several targets were decreased by 0.6 to 0.3 mg hyaluronic acid per ml but not by lesser amounts, supporting the case for a hyaluronidase-like enzyme in granulocytes as the cause of detachment of adherent target cells.

The criticism that polysaccharidases from granulocytes

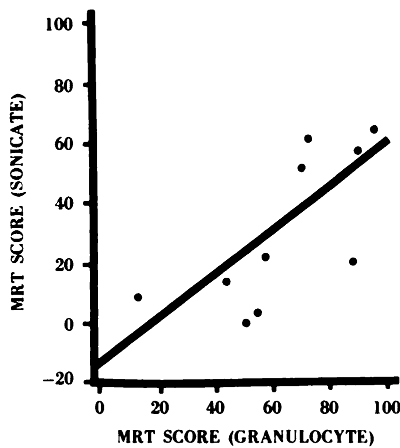

Chart 1. Granulocytes and its sonic extract from 1 person were tested against 10 different target cells. The MRT score for the sonic extract is plotted on the *ordinate*, and that for the granulocyte is plotted on the *abscissa*. The coefficient of correlation is 0.7 for a probability of 0.026.

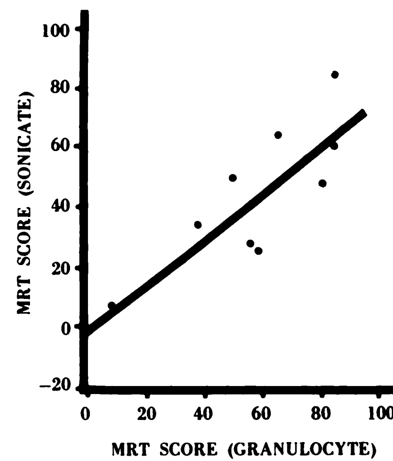

Chart 2. Granulocytes and their sonic extracts from 9 persons were tested against 1 target culture, 548. The MRT score for the sonic extracts is plotted on the *ordinate*, and that for the granulocyte is plotted on the *abscissa*. The coefficient of correlation is 0.89 for a probability of 0.001.

Table 5

Effect of granulocytes and sonically disrupted granulocytes on various target cells and inhibition by heparin

Granulocyte no.	Target no.	MRT scores with varying amounts of heparin (units/ml)						
		0.0	0.1	0.5	1.0	5.0	10.0	20.0
62308	372	77	74	73	51	55	45	35
62749	470	73	69	73	53	28	27	15
63037	370	93	94	77	61	45	35	26
63036	496	76	89	33	26	16	18	26
63037	125	70	66	58	58	29	34	30
63037	463	82	83	43	23	26	23	32
63037	805	93	91	56	19	4	0	0
Mean ± S.E.		81 ± 4	81 ± 4	59 ± 6	42 ± 7	29 ± 6	26 ± 6	24 ± 5
Sonic extract identification no.								
63968	546	52	29	22	12	0	0	0
63968	497	47	46	11	4	20	17	15
63968	133	52	52	31	14	0	3	0
63968	805	85	48	34	10	0	0	0
63968	763	70	57	31	34	27	20	12
63968	696	58	47	27	5	24	20	0
Mean ± S.E.		61 ± 6	47 ± 4	26 ± 3	13 ± 5	12 ± 5	10 ± 4	5 ± 3

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Table 6
Reversal of heparin inhibition by protamine

Target	MRT scores: No heparin	MRT scores: 10 units heparin/ml	Protamine added (mg/ml)	MRT scores: 10 units heparin + protamine/ml	MRT scores: Protamine only
462	81	7	0.05	19	91
	83	8	0.10	61	91
	86	3	0.20	69	84
124	93	18	0.05	70	94
	99	45	0.10	97	99
	93	42	0.20	95	97
133	78	13	0.05	3	50
	81	17	0.10	60	59
	81	18	0.20	43	53
497	79	31	0.05	34	56
	66	22	0.10	52	50
	67	19	0.20	63	65
697	80	0	0.05	0	80
	78	6	0.10	53	55
	76	15	0.20	62	75

Table 7
Effect of lysosomal enzymes on target cells

Target cell	No. of target cells surviving in tests, starting with 80 mg/ml, at dilutions of enzyme of: 0	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	Heparin (units/ml)
					Hyaluronidase					
463	266	0	2	4	64	133	229	298	281	0
		4	1	5	75	176	204	219	244	10
470	437	0	0	0	55	284	270	434	329	0
		3	0	1	74	228	285	416	371	10
133	622	2	3	6	12	328	668	783	675	0
		2	13	5	223	577	797	923	637	10
370	529	0	0	0	1	3	146	421	520	0
		0	2	0	3	141	303	446	486	10
					Lysozyme					
548	506	480	595	540	593	606	596	642	620	0
		518	546	600	606	593	574	555	595	10
471	115	100	98	106	105	101	106	126	113	0
		98	93	95	90	111	110	104	105	10
462	292	232	238	296	283	278	302	309	358	0
		289	295	284	313	301	300	320	297	10
372	469	506	582	702	669	652	648	655	622	0
		626	708	770	677	719	669	666	679	10
					β-Glucuronidase					
1042	38	27	35	32	40					0
471	189	152	175	174	189					0

cause nonspecific detachment of adherent target cells does not apply to tests with ^{51}Cr-labeled target cells. The sonic extract from granulocytes was tested for cytotoxicity by ^{51}Cr release at the same concentrations in which lymphocytes had previously shown strong positive reactions. The results (Table 9) indicate that sonic extracts from granulocytes, with their rich supply of enzymes, had a weak or no effect on the release of ^{51}Cr from target cells, in comparison with the effect of intact lymphocytes.

Intact and sonically treated granulocytes and lymphocytes were also tested by the chromium-release assay in the presence and absence of heparin. Only intact lymphocytes were capable of causing significant release from target cells, and heparin had no effect on this reaction. Granulocytes, whether sonically disrupted or intact, with or without heparin, had no real effect in this series.

Since the effect of granulocytes and their sonic extract is limited to the release of adherent target cells, heparin was deliberately added to tests with mixtures of lymphocytes and granulocytes to specifically nullify granulocyte reactions. Preparations containing more than 95% cells of each type were considered pure suspensions, and these cells plus

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their mixtures were tested with varying concentrations of heparin (Chart 3). When 50,000 purified granulocytes were added to the adherent target cells and incubated for 2 days, loss of target cells for both preparations approached 100% in the absence of heparin. Even with a 25% contamination of the lymphocyte suspension, a strong granulocyte effect was noticed. With pure lymphocytes, one preparation was ineffective, while the other showed a relatively strong reduction of target cells. With the addition of heparin, 0.1 unit/ml, inhibition of the granulocyte effect was noticeable and at 10 units/ml it was complete, so that only the lymphocyte-mediated cytotoxic effects remained, and target reduction correlated with the number of lymphocytes.

DISCUSSION

Since studies of cell-mediated cytotoxicity against cultured tumor cells are seeking to detect a specific immune reaction of sensitized lymphoid cells on target cells, and cytotoxic reactions are evaluated by detachment of target cells, factors that influence adherence of target cells need to be understood so that a better differentiation of specific and nonspecific effects can be made. It is well known that granulocytes from peripheral blood destroy monolayers and that this effect is counteracted by heparin (16). However, reports in the literature claim that specificity for histological-type tumor antigens was being detected with 30 to 40% granulocytes. In this study, strong reduction of some target cells was observed with even 6000 granulocytes, indicating that even a 12% contamination of the original concentration would greatly influence the final results, interfering with the detection of specificities.

Since sonic extracts from granulocytes retain the same activity, the reduction of surviving target cells by granulocytes is not the result of specific immunological sensitization but, rather, of the release of enzymes that cause detachment

Table 8

Inhibition of target cell loss with granulocytes by addition of hyaluronic acid

Granulocyte	Sonic disruption	No. of targets	MRT scores in tests with varying amounts of hyaluronic acid					
			0[a]	0.010	0.005	0.004	0.002	0.001
81530	–	4	64 ± 11			56 ± 9	68 ± 13	61 ± 11
80738	+	4	70 ± 15		51 ± 17		63 ± 15	64 ± 17
80742	+	7	55 ± 6		43 ± 7		45 ± 9	53 ± 5
80812	+	7	41 ± 6		26 ± 7		31 ± 7	36 ± 5
80875	+	7	31 ± 5	15 ± 4	26 ± 4			28 ± 6
Pooled	+	7	45 ± 6	25 ± 7	32 ± 7			35 ± 7

[a] Mg hyaluronic acid/well.

Table 9

Testing of intact and sonically disrupted effector cells by the ^{51}Cr-release assay

Target cell	Effector cell	Type of cell	Treatments: Sonic disruption	Treatments: Heparin 10 units/ml	% ^{51}Cr release
1233	78874	Lymphocyte	–	–	29
			+	–	5
			–	+	30
			+	+	–1
	78875	Lymphocyte	–	–	12
			+	–	6
			–	+	13
			+	+	–2
	78888	Granulocytes	–	–	0
			+	–	4
			–	+	–6
			+	+	–6
	78889	Granulocytes	–	–	8
			+	–	5
			–	+	0
			+	+	–10
696	78874	Lymphocyte	–	–	22
			+	–	3
			–	+	47
			+	+	2
	78888	Granulocytes	–	–	0
			+	–	9
			–	+	1
			+	+	8

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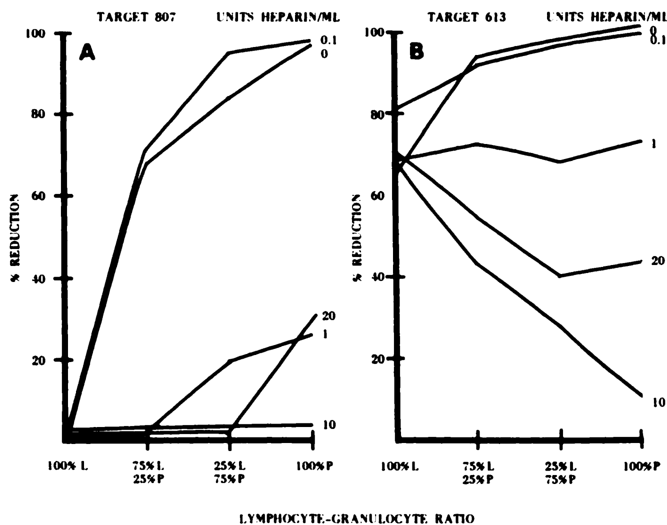

Chart 3. The effect of heparin concentrations on lymphocyte-granulocyte mixtures. Purified lymphocytes, granulocytes, and mixtures of these suspensions were tested at 50,000 cells/well on Target 613 in the presence of varying concentrations of heparin. In *A*, the lymphocyte is unreactive, whereas *B* shows a strong effect by lymphocytes.

of adherent cells. Reports that heparin was a competitive inhibitor for the enzymes hyaluronidase and lysozyme (9) led us to investigate these enzymes for their role in granulocyte-mediated detachment of target cells. Commercially prepared bovine testes hyaluronidase was a potent agent in the detachment of target cells, while lysozyme was much weaker, but neither was strongly inhibited by heparin. However, hyaluronidase is also not inhibited by heparin under physiological conditions. Small differences between the commercial enzyme from a bovine source and human granulocytic hyaluronidase under the conditions of the test might well account for differences in the effect by heparin. The increased survival of target cells with the addition of extrinsic hyaluronic acid to the sonic extract from granulocytes further suggests that a hyaluronidase-like enzyme may be the cause of the granulocyte effect.

If some enzyme is the active factor in the detachment of target cells, it is most improbable that this activity is specific for the type of tumor. Thus, a large amount of contamination of effector lymphocyte suspensions with granulocytes makes analysis of specific lymphocyte cytotoxicity impossible, since the granulocyte-mediated detachment of adherent target cells and specific immune reactions cannot be distinguished. Use of effector suspensions from healthy persons as a control for nonspecific detachment of target cells is feasible only if both effectors express exactly the same enzyme activity.

The effect of granulocytes is a problem limited to tests using detachment of cells to assess cytotoxicity and is not seen in the chromium-release assay. If such effects are easily controlled, however, the microassay using adherence of cells as a criterion of target cell viability has many advantages which are necessary prerequisites in human testing. For example, the requirement of the microtests for only minute amounts of reagents allows many replicate tests on a large battery of target cells. Electronic counting of target cells gives rapid and ready access to the effect of the lymphocytes tested. The problem of granulocyte contamination of the effector lymphocyte suspension is, of course, solved by better methods of lymphocyte separation. However, the addition of heparin to the test may also exclude granulocyte effects so that only lymphocyte-mediated cytotoxicity is detected and results are more comparable with the chromium assay.

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