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Reduced expression of distinct T-cell CD molecules by collagenase/DNase treatment

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Abstract. DNase/collagenase treatments are widely used to obtain single-cell suspensions of tumour cells and tumour-infiltrating T lymphocytes (TIL) from solid tumours. Since the functional integrity of such cells has been questioned, we have studied whether treatments with commonly used preparations of these enzymes could affect the expression of lymphocyte surface molecules and lymphocyte proliferative responsiveness. With peripheral-blood-derived T cells as a model, flow-cytometric analysis revealed strongly reduced expression of distinct CD molecules for each enzyme, notably CD2, CD4, CD8 and CD44 for DNase, and CD4, CD14, CD16, and CD56 for collagenase. The effects were found to be due to protease contaminations present in all but the purest enzyme preparations tested. Addition of serum or trypsin inhibitor abolished the effects. Since serum-free media are widely used to expand tumour-infiltrating T cells for clinical therapeutic use, data from early phenotypic analyses can be strongly misleading. Even after an 18-h rest period following the enzyme treatments, re-expression of the affected membrane markers was still far from complete. On the other hand, despite strongly reduced expression of CD2 molecules on the lymphocyte membrane, anti-CD2-induced proliferation was not affected, showing the redundancy of this signal molecule. Since other important T cell activation molecules (TCR, CD3, CD28) were not affected by enzymatic treatment, the use of expensive, highly purified collagenase/DNase preparations does not seem to be mandatory in clinical studies with expanded TIL.

Key words: Tumour-infiltrating lymphocytes – Protease contamination – CD molecules

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Introduction

Reliable tissue dissociation procedures are of pivotal importance for functional studies with tumour cells, as well as with inflammatory cells. To this end, enzymatic treatments with DNase and collagenase are widely used. In tumour immunology these enzymes have been used to obtain single tumour cell suspensions for the preparation of tumour cell vaccines in active specific immunization procedures [3, 6, 7]. More recently, DNase/collagenase treatments of tumour tissues have also been introduced for the preparation of tumour-infiltrating lymphocytes (TIL), allowing their phenotypic analysis and their expansion for passive cellular immunotherapy [2, 18, 26]. Enzymatic digestion results in better cell yields than mechanical dissociation procedures, and interleukin-2 (IL-2)-induced T cell proliferation departs most efficiently from single-cell suspensions [26].

Various digestion protocols are used by different groups, showing marked differences in enzyme concentrations and incubation times (see Table 1). Short digestion periods are assumed to be most favourable for maintenance of the condition of both tumour cells and lymphocytes, but longer incubation times and higher enzyme concentrations are frequently used to maximize cell yields. The use of fetal calf serum during the digestion period is usually avoided when cells are to be used in humans. Autologous serum could be used in therapeutic protocols, but is regularly not available in sufficient quantities. In order to prevent excessive expense, only partly purified enzyme preparations are usually used, which may contain traces of proteolytic enzymes.

Little attention has been paid thus far to the possible adverse effects of contaminating proteolytic enzymes on the phenotype and function of tumour cells and tumour-infiltrating lymphocytes. Miescher et al. [12] noted a decrease in the cell-surface expression of the CD2, CD4 and CD8 glycoproteins on lymphocytes after enzymatic dissociation with collagenase/DNase. An indication of possible adverse effects of DNase and collagenase on cell-surface expression of tumour antigens had been obtained earlier by Peters et al. [14] in the guinea-pig line 10 he-

Table 1. Dissociation protocols for isolation of tumour-infiltrating lymphocytes

Team	Medium for tumour dissociation	Serum	Time (h)	Temperature (° C)
Rosenberg et al. [18]	RPMI; 0.002%[a] DNase I, 0.1% collagenase	–	3–24	20/37
Whiteside et al. [25]	RPMI; 0.002%[a] DNase I, 0.05% collagenase	10% FCS	2	37
Yanelli et al. [26]	RPMI; 0.02%[a] DNase I, 0.1% collagenase	–	6–18	[c]
Applegate et al. [1]	RPMI; 0.04%[a] DNase I, 0.2% collagenase	–	1–1.5	[c]
Knazek et al. [8]	RPMI; 0.1%[a] DNase I, 1% collagenase	–	18	20
Baars et al. [2]	HBSS; 0.02%[b] DNase I, 0.14% collagenase	–	1–2	37

HBSS, Hanks' balanced salt solution; FCS, fetal calf serum
[a] DNase activity: 400–600 Kunitz units/mg protein
[b] DNase activity: 3000 Kunitz units/mg protein
[c] Not mentioned

patocellular carcinoma model. It was found that line 10 tumour cells obtained by enzymatic digestion were less immunogenic than line 10 cells obtained from ascites.

In the present study we took advantage of the extensive knowledge of lymphocyte surface antigens to study the effects of DNase and collagenase treatments on the expression of a large panel of membrane-bound CD molecules. Using peripheral-blood-derived mononuclear cells (PBMC) as a model, we observed markedly decreased expression of different, but not all, CD molecules after enzymatic treatment.

Materials and methods

Peripheral blood mononuclear cells. Peripheral blood mononuclear cells were derived from heparinized blood samples of healthy donors by Ficoll Hypaque density gradient centrifugation (Pharmacia, Uppsala, Sweden). The cells were collected from the interface and washed twice with phosphate-buffered saline supplemented with 1% bovine serum albumin (PBS/1% BSA, Organon Technika B.V., Boxtel, The Netherlands) and once with Hanks' balanced salt solution (HBSS, Whitaker Bioproducts, Walkerville, USA). Mononuclear cell suspensions contained 80%–90% lymphocytes and 10%–20% monocytes. Viability was over 90% as determined by Trypan blue exclusion.

Enzymatic treatments. Samples containing 10^7–2×10^7 cells were incubated at 37° C in 5 ml HBSS with DNase I (Boehringer Mannheim, lot 104132, activity 3000 Kunitz units/mg protein, and Sigma Chemicals, DN25, activity 400–600 Kunitz units/mg protein) and/or collagenase type IV (Boehringer Mannheim, lot 103578). DNase from Boehringer Mannheim was used at a concentration of 0.02% (w/v) and, in some experiments, DNase from Sigma was used at 0.1% (w/v) corresponding to an enzyme activity of 400–600 Kunitz units/ml medium. RNase-free DNase I (f-DNase), checked for the absence of proteases, was used from Boehringer Mannheim (lot 776785). Collagenase was used at a concentration of 0.14% (w/v), corresponding to 0.2 U/ml. Control incubations were in HBSS alone. Where indicated, heat-inactivated fetal calf serum (FCS; Gibco, Paisley, Scotland) or protease inhibitors were added during the enzymatic digestion. Every 15 min the cell suspensions were gently agitated. After 2 h the cells were washed five times in HBSS or phosphate-buffered saline and used for further experiments.

Protease inhibitors leupeptin (Boehringer Mannheim, lot 1017101), pepstatin (Boehringer Mannheim, lot 243286) and trypsin inhibitor from Sigma Chemicals (T9253) were added in concentrations of 1 μg/ml or 10 μg/ml. The trypsin inhibitor preparation used contained both ovoinhibitor and ovomucoid, known to inhibit activities of several proteases including both trypsin and chymotrypsin [5, 10].

Antibodies. Unconjugated antibodies against CD16 (Leu11b), CD25 (Tac), CD56 (Leu19), conjugated antibodies against CD3 (Leu4-FITC), CD4 (Leu3-FITC), CD8 (Leu2a-PE), CD14 (LeuM3-PE), CD19 (Leu12-PE), HLA-DR [anti-(HLA-DR)-FITC], CD45 (LCA-FITC) and the isotype controls [IgG1-FITC, IgG2a-PE] were purchased from Becton Dickinson. RIV6 (anti-CD4) and RIV4 (anti-CD8, clone WT82) were obtained from the RIVM (Bilthoven, The Netherlands). W6/32 (anti-HLA-I) was obtained from Seralab. F10.2 is directed against ICAM-1 [19] and was kindly provided by Dr. A. C. Bloem. HP2/1, directed against VLA-4 [20], was kindly provided by F. Sánchez-Madrid. TB133 is directed against LFA-1α (CD18/CD11a) [24]. NKI-P2 (anti-CD44) and WT31 (anti-TCR) were a kind gift from Dr. C. Figdor (NKI, Amsterdam, NL) and Dr. W. Tax (Radboud Hospital, Nijmegen, NL) respectively. Antibodies against CD3 (16A9), CD28 (15E8), CD45 (15D9) and several distinct epitopes of CD2 (HIK27, 6G4, 4B2) were kindly provided by Dr. R. A. W. van Lier (CLB, Amsterdam, NL). Fluorescein-isothiocyanate(FITC)-labelled conjugate [rabbit anti-(mouse IgG)-FITC] and phycoerythrin(PE)-labelled conjugate [goat anti-(mouse IgG)-PE] were purchased from Dakopatts (Glostrup, Denmark).

FACS analysis. Preceding the immunofluorescence staining the cells were washed once in PBS/1% BSA supplemented with 0.02% NaN_3 (Merck, Darmstadt, Germany). For direct immunofluorescence assays, aliquots of approximately 0.25×10^6 cells were incubated with 25 μl PE- or FITC-labelled monoclonal antibodies for 30 min. Cells were washed with PBS/1% BSA with NaN_3, resuspended in 0.5 ml same solution and stored at 4° C until fluorescence-activated cell sorting (FACS) analysis.

For indirect immunofluorescence, aliquots of approximately 0.25×10^6 cells were preincubated in 25 μl normal rabbit serum (Dako, Glostrup, Denmark) for 20 min and 25 μl monoclonal antibody was added. After 30 min the cells were washed once in PBS/1% BSA with 0.02% NaN_3, and resuspended in 25 μl FITC labelled conjugate. After a 30-min incubation the cells were washed and resuspended in 0.5 ml PBS/1% BSA with 0.02% NaN_3. All incubations were done at room temperature.

Fluorescence analysis was done within 24 h after staining, by FACS (FACSTAR plus, Becton Dickinson, Mountain View, Calif., USA). A decrease in fluorescence intensity was calculated as

$$\frac{\text{fluorescence intensity of the positive peak of the enzyme-treated sample}}{\text{fluorescence intensity of the positive peak in the medium control}} \times 100\%$$

Statistical significances were calculated using the Mann-Whitney *U*-test.

Re-expression of membrane markers. Following enzymatic treatment, the cells were washed five times and resuspended in either HBSS or in RPMI-1640 medium supplemented with 5% FCS with or without 10 μg/ml phytohaemagglutinin (Wellcome Diagnostics, Dartford, UK). After 0, 30, 60, 120 min and 18 h incubation at 37° C, 5% CO_2, an aliquot of cells was washed in PBS/1% BSA with 0.02% NaN_3, and stored at 4° C until immunofluorescence staining.

Proliferation assays. Microtiter plates (96 wells) (Greiner, Alphen a/d Rijn, The Netherlands) were coated with anti-CD3 or anti-CD2 (1 μg/

Table 2. Expression of human peripheral blood lymphocyte (PBL) membrane markers after treatment with DNase or collagenase

Marker	Antibody	DNase	(*n*)	Collagenase	(*n*)
CD3	Leu4	=	(9)	=	(2)
	16A9	=	(9)	=	(3)
TCR	WT31	=	(3)	ND	
CD2	HIK27	↓↓↓*	(9)	=	(2)
	6G4	↓↓↓*	(28)	=	(12)
	4B2	↓↓↓*	(9)	=	(2)
CD4	Leu3	↓↓↓*	(25)	↓↓*	(10)
	RIV6	↓↓↓**	(4)	ND	
CD8	Leu2a	↓↓↓*	(28)	↓**	(8)
	RIV4	↓↓↓	(3)	ND	
CD14	LeuM3	↓↓↓*	(9)	↓↓	(2)
CD16	Leu11b	↓	(6)	↓↓	(2)
CD19	Leu12	=	(6)	=	(2)
CD25	Tac	=	(5)	ND	
CD28	15E8	↓	(9)	=	(2)
CD44	NKI-P2	↓↓↓*	(27)	=	(13)
CD45	LCA	=	(28)	=	(12)
	15D9	=	(6)	ND	
CD56	Leu19	↓	(7)	↓↓	(2)
HLA class I	W6/32	=	(8)	=	(2)
HLA DR		=	(9)	=	(2)
ICAM-1	F10.2	=	(6)	ND	
LFA-1α	TB133	=	(9)	=	(2)
VLA-4	HP2/1	=	(4)	ND	

Human PBL were treated for 2 h with the indicated enzymes. The concentrations (w/v) of the different enzymes were 0.1% for DNase from Sigma, 0.02% for DNase from Boehringer, and 0.14% for collagenase. The number of donors is indicated within brackets. The change in fluorescence intensity is given as the percentage reduction of the peak fluorescence intensity compared to cells treated with HBSS alone: =, increase or decrease of less than 25%; ↓, decrease of more than 25% but less than 50%; ↓↓, decrease of more than 50% but less than 75%; ↓↓↓, decrease of more than 75%.
* $P < 0.001$
** $P < 0.05$

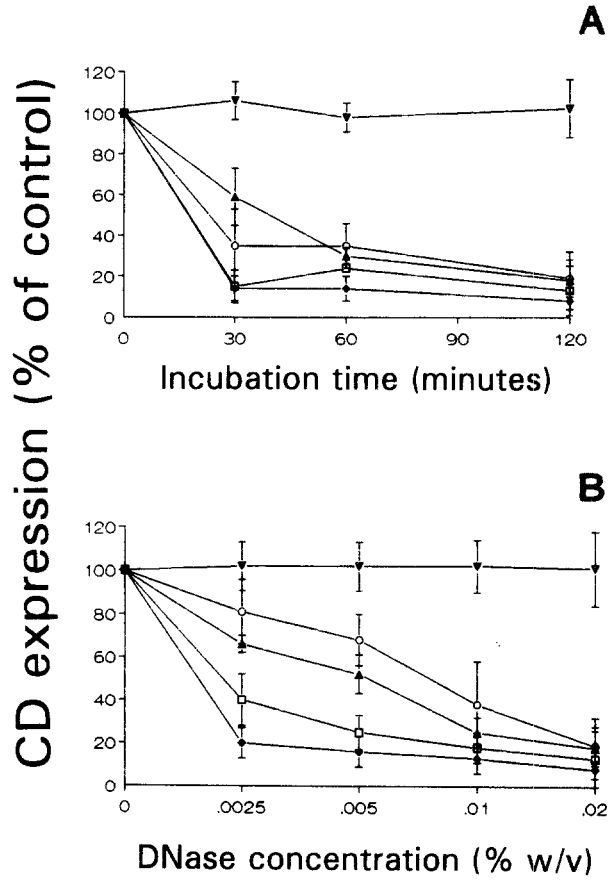

Fig. 1A, B. Decay of cell-surface molecule expression after DNase I treatment is dependent on incubation time and DNase concentration. Peripheral blood mononuclear cells (PBMC) were incubated for 0, 30, 60 or 120 min in Hanks' balanced salt solution (HBSS) containing 0.02% DNase, corresponding to 400–600 Kunitz units/ml enzyme activity (**A**) or for 120 min in HBSS containing different concentrations of DNase (Boehringer) (**B**). Following incubation, cells were washed five times with HBSS and once with phosphate-buffered saline (PBS) 0.02% NaN_3, stained by indirect immunofluorescence and analysed for expression of CD45 (▼), CD2 (▲), CD4 (○), CD8 (◆) and CD44 (□). Data from one representative experiment are given

ml) in sterile PBS for 18 h at 4° C. Before use the plates were washed three times with sterile PBS. Samples containing 5×10^4 PBMC were cultured in 100 µl medium supplemented with 10 IU/ml IL-2 (Cetus Corporation, Emeryville, Calif., USA) and 1 µg/ml anti-CD28 where indicated. In the case of stimulation with immobilized anti-CD2, the microtiter plate was coated (1 : 1000) with one of the three monoclonal antibodies against CD2, and the remaining two antibodies were added at a 1 : 1000 dilution during the culture. Proliferation was determined after 3 days of culture by measuring the incorporation of [^{3}H]thymidine (14.8 Bq/well; Amersham, Amersham, UK) added 4 h before harvest of the culture. Radioactivities (cpm) are given as the means ± SD of triplicate cultures. Statistical significances were calculated using the Wilcoxon rank-sum test.

Results

Cell-surface antigen expression after enzymatic treatments

DNase treatment of PBMC profoundly reduces the cell-surface expression of CD2, CD4, CD8, CD14 and CD44 antigens (exceeding 75% reduction as measured by fluorescence intensity; Table 2). Expression of CD16, CD28 and CD56 was diminished to a marked less extent (25%–50% reduction). DNase from Boehringer and from Sigma showed identical effects. Collagenase treatment of lymphoid cells also led to a marked reduction of expression of cell-surface molecules. Interestingly, the pattern of reduction was very different from that observed with DNase treatment, with CD4, CD14, CD16 and CD56 molecules being most sensitive (50%–75% reduction).

For CD2, CD4 and CD8 we investigated whether the observed decrease in expression after DNase treatment was limited to distinct epitopes on the antigens. Sets of monoclonal antibodies to CD2, CD4 and CD8 were used that recognize non-overlapping epitopes [9, 15, 23]. As can be seen in Table 2, for each CD molecule the reduction of expression was identical for all epitopes, indicating that large parts of the molecules had disappeared from the lymphocyte membranes.

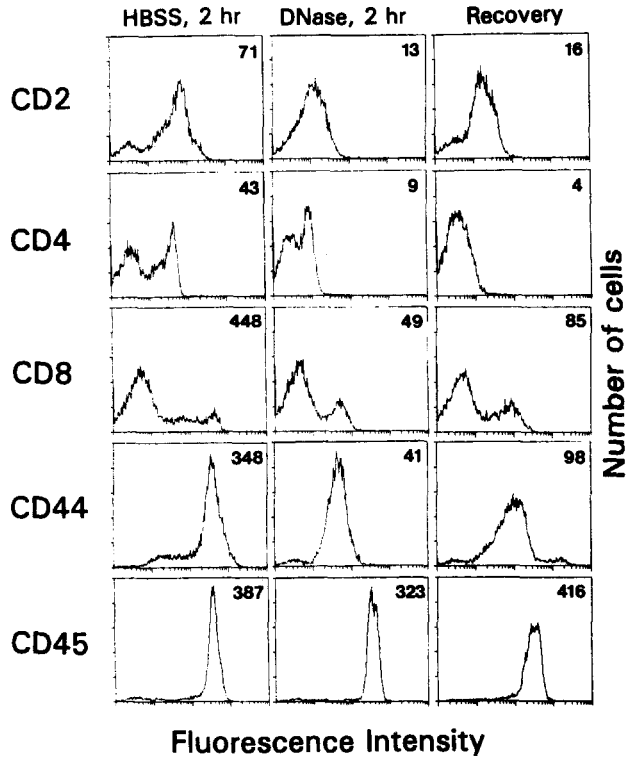

Fig. 2. Reduction of expression of various CD molecules on PBMC after enzymatic treatment with DNase (*middle column*) or control treatment (HBSS, *left column*). Expression is only slightly restored after an 18-h recovery period (*right column*). Numbers represent the fluorescence intensities for the respective peaks. Data from one representative experiment are given

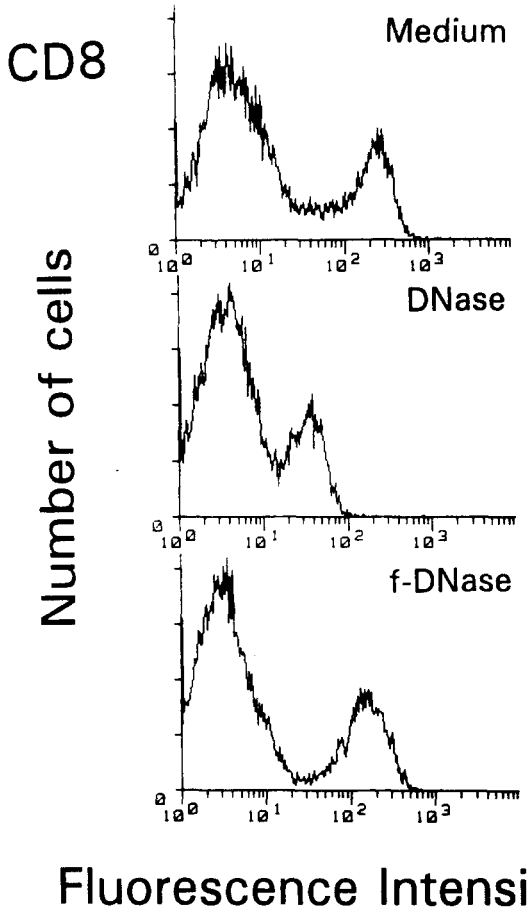

Fig. 3. Expression of the CD8 molecule is not affected by treatment with highly purified, protease-free DNase. PBMC were incubated for 2 h in HBSS alone, in HBSS with 0.02% DNase or in HBSS with protease-free DNase (*f-DNase*). Data from one representative experiment are given

The effect of DNase was dependent on the time of incubation. Figure 1 A shows that for most CD molecules 30 min of enzymatic treatment led to almost maximal reduction in cell-surface expression. As expected, the effect was also found to be dose-dependent (Fig. 1 B). It is important to note that even with the lowest reported dose of DNase (0.002%, corresponding to approximately 8–12 Kunitz units/ml [18]), marker expression was severely decreased when cells were incubated for 18 h (85%–95% reduction; data not shown).

Re-expression of affected markers

It was possible that restoration of the expression of the affected membrane markers could be induced by incubating the cells at 37° C after enzymatic treatment. Figure 2 shows a distinct, but far from complete, re-expression of CD2, CD4, CD8 and CD44 molecules during the 18-h recovery period. Recovery in the presence of phytohaemagglutinin did accelerate the expression of CD44 and CD2, but only the expression of CD2 was fully restored within 18 h (data not shown).

Effects of protease inhibitors

Three different protease inhibitors were tested for their possible capacity to prevent the decrease in expression of the membrane glycoproteins. Whereas leupeptin and pepstatin had no significant effects, trypsin inhibitor prevented the effect of DNase treatment for all sensitive CD molecules. FCS, known to contain antiproteolytic proteins, could, when present during enzyme treatment, also prevent the decrease in expression of CD2, CD4, CD8 and CD44. Notably, addition of FCS did not affect DNase or collagenase enzyme activities (data not shown). With a highly purified preparation of DNase, no decrease in expression of CD2, CD4, CD8 and CD44 was observed (for CD8, see Fig. 3), confirming that contaminating proteolytic enzymes were most likely causing the reduction of CD expression.

T cell proliferation after enzymatic treatment

T lymphocyte proliferation can be stimulated along different pathways, notably involving the CD2, CD3 and CD28 surface molecules. Since CD2 molecule expression was particularly sensitive to enzymatic treatment, we studied whether DNase pretreatment of PBMC might interfere with CD2-ligand-induced proliferative responses. Anti-CD28 was included in the media to provide an effective costimulatory signal [4, 16, 23]. Figure 4 shows that the

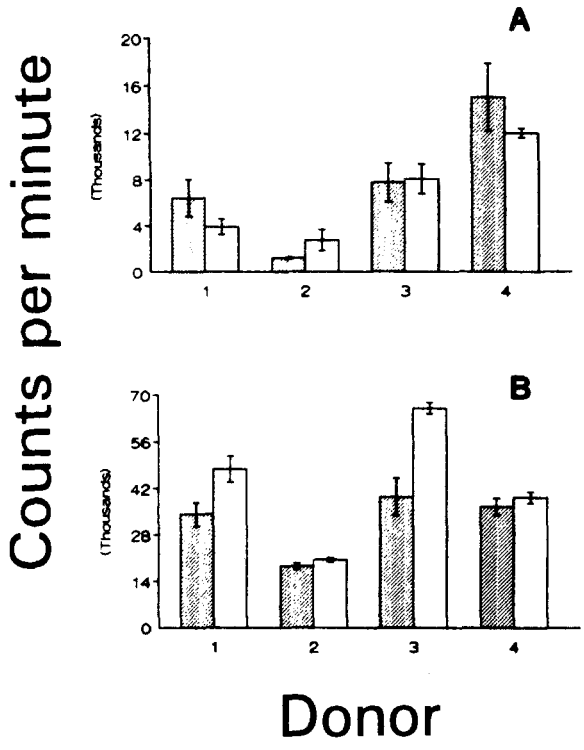

Fig. 4A, B. DNase treatment of PBMC does not interfere with anti-CD2- and anti-CD3-driven T cell proliferation, as measured by [^{3}H]thymidine incorporation. PBMC from healthy donors were incubated with HBSS (*shaded bars*) or 0.02% DNase (*open bars*) for 2 h, washed and subsequently cultured with anti-CD2 (**A**) or anti-CD3 (**B**) monoclonal antibodies for 3 days. Means of triplicate cultures are given for four different donors. Background values for cultures without antiCD2 or antiCD3 antibodies were always below 200 cpm

anti-CD2-driven proliferation of DNase-treated PBMC from four different donors was not reduced. As expected, CD3-induced proliferative responses were also not impaired by DNase treatment. In fact, for two out of four donors a slight increase in proliferation of DNase-treated cells was seen as compared to the control cells.

Discussion

In this study we show that enzymatic treatment of PBMC with DNase and/or collagenase strongly reduces the cell-surface membrane expression of several membrane glycoproteins. Involvement of contaminating protease(s) was demonstrated. Strikingly, DNase preparations from two different commercial sources showed identical patterns in CD molecule reductions, suggesting the presence of a major contaminating protease recognizing shared amino acid sequences or conformational sites. A similar, but distinct, protease affecting the expression of limited cell-surface antigens has recently been described by Sutherland et al. [22].

The present findings bear particular relevance to phenotyping studies on cell suspensions obtained from enzymatically dissociated solid tissues. The 16-h incubation period, frequently used before phenotypic analysis or functional tests are performed [12, 21], is not sufficient to restore fully the expression of all affected markers. The actual composition of such solid-tissue-derived cell suspensions may be seriously misjudged when the present observations are not taken into account. This may in particular hold for T cell subset estimations based on CD4 and CD8 data, but analysis of tumour-infiltrating lymphocytes for expression of adhesion molecules like CD44, which in murine models are associated with a protective capacity of CD8$^+$ T lymphocytes [17], would also suffer from enzymatic digestion procedures to obtain the TIL. Nevertheless, since reduction of expression of the CD molecules never reached 100% values, dimly staining cells might still allow the correct percentages of lymphoid subsets to be estimated. We observed a decrease in the percentage of positively staining cells (from 30% to 10%) after collagenase treatment only for CD56. In line with this view, Newman [13] recently reported that brief exposure to 12.5 U/ml collagenase (30 min at 37° C), to separate migrating lymphocytes out of a collagen gel, did not affect the percentages of cells scored positive for, amongst others, CD4 and CD8. Notably, no data on fluorescence intensities were given.

Our present study has focused on the expression of CD molecules on lymphoid cells. When tumour cell suspensions are being prepared for vaccine purposes, however, similar deleterious effects on distinct tumour-associated surface molecules may be expected. Thus, individual tumour cell antigens could also be affected by enzymatic dissociation procedures, and ultimately lead to lower vaccine efficiencies. Earlier observations in an experimental tumour vaccination model clearly support this view [14].

The only major cell-activation pathway affected by enzymatic treatment involved the CD2 receptor. The severe reduction of CD2 expression on DNase-treated T cells did not, however, result in a significant impairment of anti-CD2-induced proliferation. This finding may be explained by the redundancy of CD2 molecules on the cell membrane, the remaining numbers of molecules being sufficient to transduce the proliferative signal and cause T cell proliferation. Moreover, activation of lymphocytes may lead to a rapid up-regulation of expression of CD2 molecules on the cell membrane [11]. In accordance with the finding that expression of neither CD3 nor T cell receptor molecules was affected by enzymatic treatment, no impairment of anti-CD3-induced proliferation could be detected. Collectively, these results make it clear that enzymatic dissociation of solid tissues is unlikely to impair subsequent T cell proliferative capacities.

In conclusion, for the enzymatic preparation of solid-tissue-derived cell suspensions (autologous) serum-containing media, or, alternatively, ultrapure enzyme preparations should be used. These provide optimal conditions for full retention of lymphoid cell-surface molecules, allowing correct analysis or quantification of the tissue-infiltrating cells. When these conditions are not met, however, reduced expression of membrane molecules does not necessarily interfere with the proliferative responsiveness of infiltrating T cells.

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