Eur J Cancer, Vol. 28A, No. 4/5, pp. 749–754, 1992.
Printed in Great Britain

0964–1947/92 $5.00 + 0.00

Signalling in Human Tumour Infiltrating Lymphocytes: the CD28 Molecule is Functional and is Physically Associated with the CD45R0 Molecule

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The CD28 T cell activation pathway was functional in human tumour infiltrating lymphocytes (TIL) and can induce strong proliferation, lymphokine release and calcium mobilisation. Conversely, TIL responded poorly to stimulation via CD2, and CD28 did not synergise with CD2, which is at variance with that observed using peripheral lymphocytes from the same patients. On stimulation with anti-CD28 the monoclonal antibody, most TILs, which were CD3$^+$, CD28$^+$ and CD45R0$^+$ at the beginning of culture, co-expressed both high (CD45RA) and low (CD45R0) molecular weight isoforms of CD45. CD28 was associated with the CD45R0 isoform at the cell surface of activated TIL, as demonstrated by immunoprecipitation and immunoenzymatic assay. Thus CD28 can substitute for CD3 in TIL leading to the expansion of functional lymphocytes and to the amplification of antitumour immune response.
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INTRODUCTION

BECAUSE OF the demonstration of their potent antitumour activity in *in vivo* murine models [1], human tumour infiltrating lymphocytes (TILs) have been extensively studied. The possible adoptive transfer of *ex vivo* activated TILs, to improve the efficacy of immunotherapy in human cancer [2], has been hampered by the fact that human TILs are not easily activated *in vitro* via the antigen-dependent CD3/T cell receptor (TCR) pathway [3]. Antigen-specific cell-contacts in the immune system are strengthened by antigen non-specific interactions, such as pairing of CD2 and CD28 with their ligands [4, 5]. Moreover, the antigen-independent pathways of T cell activation mediated by CD2 and CD28 molecules could function in CD3-unresponsive T lymphocytes [6]. In the latter [6], CD2- and CD28-induced cell proliferation was accompanied by the loss of the high molecular weight isoform of CD45 (HWM, CD45RA) and increased expression of the low molecular weight (LMW) CD45R0 isoform. The differential expression of CD45RA and CD45R0 isoforms has been claimed by some authors to define two non-overlapping T cell subsets containing naive and memory cells, respectively [7]; however, controversial data have been reported on this argument [8, 9]. These considerations prompted us to study the *in vitro* response of human TILs to triggering via CD2 or CD28, and the regulatory mechanisms possibly modulating their function. Indeed, one might speculate that, at the tumour site, receptor–ligand interactions other than TCR–antigen recognition take place, thus amplifying or substituting for CD3-mediated activation. As TILs are localised at the site of the hypothetical antigen, they should display the surface phenotype of memory T cells (CD45R0$^+$), according to data from other laboratories [10]. Several reports have indicated that CD45 molecule could regulate lymphocyte activation due to a tyrosine phosphatase activity linked to its cytoplasmic domain [11–14]. For these reasons the analysis of different CD45 isoforms related to the T cell activation pathways should be of interest in the study of TILs function. In this paper we investigated whether CD2 or CD28 can substitute for CD3 in TILs activation and whether physical association between certain isoforms of CD45 and T cell activation pathways accounts for the functional behaviour of TILs.

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Revised 29 Nov. 1991; accepted 31 Dec. 1991.

MATERIALS AND METHODS

Monoclonal antibodies

Fluorescein (FITC) or phycoerythrin (PE) conjugated monoclonal antibodies (Mabs) directed against CD2 (Leu5b), CD3 (Leu4), CD56 (Leu19), WT31 (TC 31), CD45RA (Leu18), and purified anti-CD7 (Leu9) and anti-CD5 (Leu1) were all purchased from Becton Dickinson, anti-CD45R0 Mab (UCHLI) from Unipath (Milan); FITC-goat antimouse antiserum from Southern Biotech. (Birmingham, Alabama). For functional studies the anti-CD3 JT3A [15], the anti-CD28 9.3 (Coulter Immunology) or Kolt2 (Janssen) or CK248 [16] Mab and the anti-CD2 pair CD2.1 and CD2.9 [17] were used.

Cell preparation

Peripheral blood lymphocytes (PBLs) were isolated by discontinuous density gradient centrifugation. Autologous fresh tumour biopsy specimens were obtained at surgery from 6 primary lung tumours and 6 renal cell carcinomas [18]. Tissues were digested with DNAse 0.002%, collagenase 0.1% and hyaluronidase 0.01% (Sigma) for 3–4 h at 37°C, washed and

enriched in either TILs or tumour cells on Ficoll–Hypaque differential gradient (100% and 75%). The enzymatic treatment did not affect either the functional capabilities or the expression of surface cell markers, as assessed by determining cell proliferation and surface phenotype of PBL, after incubation for 3 h with the same enzymatic mixture used for digesting tumour specimens (not shown). TILs were seeded in 96 wells U bottom plates (10^5 cells per well) and stimulated with anti-CD28 and/or anti-CD2 Mab in the presence of either allogeneic spleen cells or autologous irradiated PBL (10^5 per well) as a source of feeder cells. After 48 h, recombinant interleukin-2 30 U/ml (rIL2, Eurocetus) was added to the culture medium (RPMI 1640); Biochrom, supplemented with antibiotics, L-glutamine and 10% fetal calf serum (Biochrom). Under these culture conditions cells grew for at least 3–4 weeks and phenotypic analysis was performed at different time points.

Phenotypic analysis of TILs

The surface phenotype of either fresh or cultured TILs was determined by two-colour cytofluorometric analysis as described [15]. Briefly, aliquots of 5×10^5 cells were harvested at day 4, 10, 17 or 24, stained with the corresponding Mab, washed and analysed on a fluorescence-activated cell sorter (FACStar, Becton Dickinson) gated to exclude non-viable cells. Results are expressed on the contour plot as log green versus log red fluorescence intensity and are referred to 1 out of 12 independent experiments (6 with TIL from lung carcinoma and 6 from renal cell carcinomas).

Proliferation assay

TIL or PBL isolated from the same patients were cultured in triplicate wells (10^5 cells per well) in culture medium and stimulated with 5 μg per 10^6 cells of either anti-CD3, or anti-CD28, or anti-CD2 (CD2.1 plus CD2.9) Mabs in the presence of irradiated feeder cells (see above). In some experiments rIL2 was added after 48 h. In two cases TILs were fractionated into $CD45RA^-$ and $CD45R0^-$ by negative depletion with anti-CD45RA or anti-CD45R0 Mab using immunomagnetic beads (Unipath). Highly purified (> 99%) $CD45RA^-$ and $CD45R0^-$ populations were then used to determine the proliferative response to anti-CD28 and anti-CD2 Mabs. DNA synthesis was measured at 24, 48, 72 and 96 h by pulsing the cultures with 37 kBq 3H-methyl-thymidine (Amersham) 18 h before harvesting. Triplicate samples were then counted in a β-counter (Packard Instruments). Results are expressed as c.p.m. and represent the mean values (S.D.) of 12 experiments with TILs or PBLs derived from 6 patient with renal cell carinoma and 6 with lung adenocarcinoma.

TNF-α and IFN-γ production assay

After 48 h, aliquots of cell free supernatants were removed from TIL cultures triggered with the various simulating Mabs and used for determination of tumour necrosis factor (TNF-α) or interferon (IFN-γ) production [19]. IFN-γ units were determined using vesicular stomatitis virus-infected human amniotic cells in 50% cytopathic effect reduction assay. IFN-γ titres present in the supernatant were evaluated as the reciprocal of the highest dilution giving 50% reduction of cytopathic effect in infected control cultures. Units per ml of IFN-γ were calculated in comparison with IFN-γ titres of a standard curve. TNF-α activity was tested evaluating the cytopathic effect of supernatant from TIL on actinomycin-D (Sigma) treated WEHI 164 sarcoma cells in a ^{51}Cr release assay. Supernatant-induced specific lysis of ^{51}Cr-labelled WEHI 164 cells was compared with lysis obtained using purified TNF-α (T Cell Sciences). Picograms per ml of TNF-α were calculated on the basis of a standard curve.

Calcium mobilisation studies

Proliferating TILs (1.5×10^6/ml), were loaded with the acetoxymethyl ester of Fura-2 (1 μmol/l final concentration, Sigma) and triggered with either anti-CD2 or anti-CD28 Mab. The fluorescence of the cells was monitored with a spectrofluorimeter (LS5, Perkin Elmer) using 2 ml quartz cuvettes. The cell suspension was excited at 340–380 nm and fluorescence was measured at 495 nm. Five slit widths were used for both excitation and emission. All measurements were performed at 37°C using a thermostatically controlled cuvette holder and stirring apparatus. Intracellular calcium concentration ($[Ca^{2+}]_i$) was calculated by the method of Grynkiewicz [20].

Immunoprecipitation of the CD28 and CD45R0 molecules

Cells were surface labeled with ^{125}I using lactoperoxidase catalysed iodination, washed and lysed in 10 mmol/l Tris-buffered saline (pH 7.5) containing 0.3% CHAPS (Sigma) in order to avoid disruption of non-covalently associated molecules on the cell membrane. After spinning at 10 000 *g*, lysates were precleared with protein-A Sepharose beads (PAS, Pharmacia-LKB) and immunoprecipitated with either anti-CD28 or anti-CD45R0 and PAS. The immunoprecipitates were eluted by boiling in 100 mmol/l Tris, 2% sodium-dodecyl-sulphate (SDS) and analysed on a 4–12% SDS–polyacrylamide gel gradient (PAGE).

Solid-phase ELISA

Immunoprecipitation was performed in 96-well microtitre ELISA plates coated with either 1 μg of either Kolt2 (IgG1) or 9.3 (IgG2a) anti-CD28 Mab or 1 μg of anti-CD45R0 Mab (IgG2a). Lysates (10^6 cells per well) were incubated for 2 h at room temperature. After immunoprecipitation, plates were washed four times with 200 μl lysis buffer and immunoprecipitates incubated for 1 h with 100 μl of anti-CD5 or anti-CD7 (IgG1) or anti-CD28 or anti-CD45R0 Mab (10 μg/ml in phosphate buffered saline [PBS], 1% bovine serum albumin [BSA]). Plates were washed three times with 200 μl PBS plus 1% BSA and 100 μl of biotin-labelled specific goat antimouse antiserum (1:1000 dilution, Amersham) was added. After 1 h, plates were washed and incubated with 100 μl avidin-peroxidase (Amersham) for 45 min. Then 100 μl substrate buffer containing diaminobenzidine was added and adsorbance was determined at $\lambda = 492$ nm using a Multiscan (Perkin–Elmer).

RESULTS

Expression of CD45R0 isoform by TILs

TILs derived from both lung tumours and renal cell carcinomas were mainly $CD28^+$ and coexpressed the CD3/TCR complex (> 90%; Fig. 1a), whereas a lesser fraction (10–15%) was $CD28^-CD3^-CD56^+$ (not shown). Most of the $CD3^+CD28^+$ cells reacted with the UCHLI Mab (Fig. 1b and c); thus, the majority of TIL might show a functional behaviour similar to that of memory T cells. It is of note that less than 5% of fresh TIL coexpressed the CD45R0 and the CD45RA isoforms (Fig. 1d); however, in some cases (2 out of 6 lung tumours and 1 out of 6 renal cell carcinomas), 15–30% of TIL were both $CD45R0^+$ and $CD45RA^+$ at the beginning of the culture (Fig. 2a).

CD28-induced proliferation of TILs

TILs obtained from both kidney (Table 1) and lung (Table 2) tumours proliferated after stimulation via CD28 as assessed by 3HTdR uptake. In contrast, they gave a poor response to anti-CD2. Little or no proliferation was observed when TILs were triggered via CD3. In some experiments the addition of exogenous rIL2 to CD2-stimulated, but not to CD3-stimulated cultures led to a slight increase in the proliferation rate (not shown). The observation that CD28 but not CD2 activation pathway was fully functional in TILs was further supported by the finding that no synergism between the two activation pathways was observed (Tables 1 and 2). Note that PBLs derived from the same patients exhibited a good response to all the stimuli used, including CD2. Moreover, in this case synergism between CD28 and CD2 activation pathways was evident (Tables 1 and 2). It is of interest that, as demonstrated by sorting experiments, the TIL population which responded to CD28-induced activation was $CD45R0^+$. Indeed, $CD45RA^-$ TILs, obtained by negative depletion in order to avoid regulating signals transduced via CD45, showed a good proliferation to anti-CD28 (Table 3) and were all $CD45R0^+$ (not shown). On the other hand $CD45R0^-$ cells gave only a slight proliferative response to stimulation via CD28.

To define whether TILs could release soluble factors in response to stimulation via CD28 or CD2, TNF-α and IFN-γ were quantitated in supernatants collected after stimulation. As shown in Table 4, TILs released large amounts of IFN-γ in response to both Mabs; on the other hand, TNF-α was mainly produced after stimulation via CD2. It is of note that the two pathways worked synergistically in inducing TNF-α production by TIL from renal cell carcinomas.

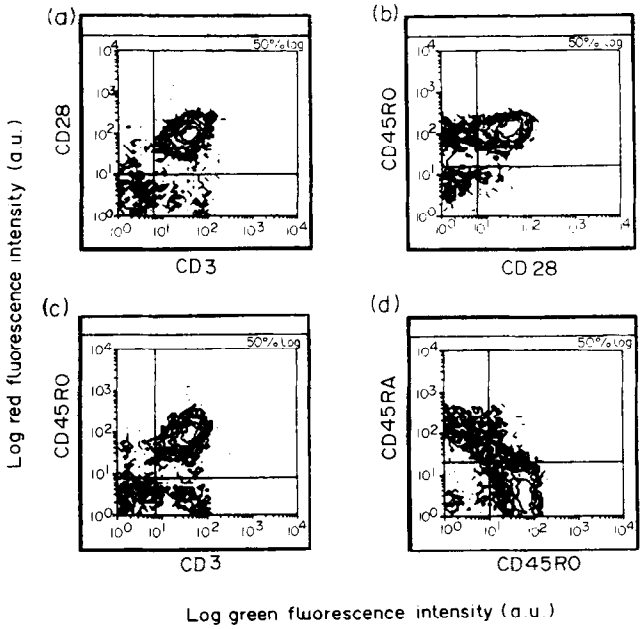

Fig. 1. Surface phenotype of fresh TILs. TIL population recovered from the 100% Ficoll fraction, was pure at 96–99% and the number of cells varied depending on the tumour size (from 15 to 25 × 10_6). No difference in cell recovery or in surface phenotype was found between lung adenocarcinomas and renal cell carcinomas. Contour plots represent log green fluorescence intensity (*x*-axis) vs. log red fluorescence intensity (*y*-axis). Panel (a): CD3 vs. CD28, panel (b): CD28 vs. CD45R0, panel (c): CD3 vs. CD45R0, panel (d): CD45R0 vs. CD45RA. Samples were run on a FACStar Plus. Results are representative of 6 independent experiments with TILs from renal cell carcinomas and 6 with TIL from lung adenocarcinomas.

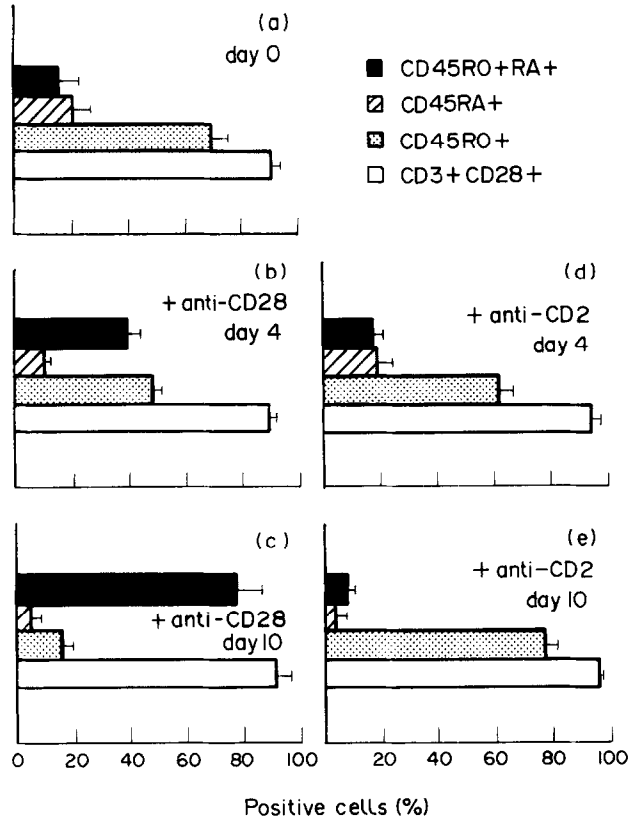

Fig. 2. Kinetics of CD45RA and CD45R0 expression on human TILs at day 0 (a), day 4 (b, d) and day 10 (c, e) of culture. Cells were isolated from both lung and renal cell carcinomas and stained with the specific Mab after stimulation via CD28 (b, c) or CD2 (d, e). Results are expressed as percentage of positive cells (S.D.) from 12 experiments (6 with TILs from lung tumours and 6 from renal cell carcinomas).

Preferential growth of $CD45R0^+RA^+$ TILS after stimulation via CD28

Upon stimulation of TILs via either CD28 or CD2 and subsequent culture in the presence of rIL2, growing cells were all $CD3^+CD28^+$ (Fig. 2). It is noteworthy that the percentage of cells coexpressing the CD45R0 and RA isoforms increased (up to 90%) during the culture after stimulation via CD28 (Fig. 2b, c) but not via CD2 (Fig. 2d, e). On the other hand, CD2-activated TILs were mostly $CD45R0^+RA^-$ (Fig. 2d, e). In several cases proliferating T cell lines were derived from $CD28^+$ activated and, to a lesser extent, CD2-activated TILs and used for calcium mobilisation studies.

CD28-induced calcium mobilisation in cultured TILs

When FURA2-loaded $CD3^+$ activated TILs were challenged with purified anti-CD28, a strong and early calcium mobilisation was observed without the need of surface cross-linking (Fig. 3d). By contrast, addition of anti-CD2 antibodies had no effect (Fig. 3e), even after cross-linking with goat antimouse antiserum (not shown). Conversely, a detectable $[Ca^{2+}]_i$ increase was evoked via CD2 in PBL from the same patients (Fig. 3b). In all the experiments the subsequent addition of anti-CD2 and anti-CD28 showed that no synergistic effect between the two path-

Table 1. Proliferative response of TILs and PBLs from 6 patients with renal cell carcinomas

Cells	Stimuli	24 h	48 h	72 h	96 h
TIL	None	0.9 (0.1)	1.8 (0.5)	1.9 (0.4)	1.9 (1.2)
	anti-CD28	2.8 (1.2)	10.1 (4.2)	16.1 (5.8)	20.1 (11.8)
	anti-CD2	1.1 (0.4)	3.0 (0.2)	2.5 (0.3)	2.9 (0.7)
	anti-CD3	1.5 (0.5)	3.3 (0.6)	2.4 (0.6)	2.1 (0.2)
	anti-CD2+ anti-CD28	2.1 (0.9)	9.6 (1.0)	18.1 (3.0)	24.6 (10.9)
	rIL2	ND	ND	2.2 (1.4)	2.0 (0.8)
Feeder cells alone		0.7 (0.2)	1.8 (0.4)	1.0 (0.9)	1.7 (1.5)
PBL	None	0.5 (0.2)	1.0 (0.3)	2.0 (0.5)	1.8 (0.4)
	anti-CD28	2.1 (0.4)	5.2 (2.4)	20.5 (6.1)	37.5 (5.2)
	anti-CD2	1.7 (0.8)	10.5 (4.3)	19.7 (2.9)	45.7 (3.7)
	anti-CD3	22.1 (10.4)	115.7 (10.8)	89.7 (3.8)	13.6 (4.6)
	anti-CD2+ anti-CD28	2.9 (1.0)	35.6 (4.8)	66.4 (2.9)	109.2 (8.8)
	rIL2	ND	ND	11.6 (4.0)	12.4 (6.0)

Freshly isolated TILs and PBLs were stimulated for the indicated periods of time with the various Mabs. ^{3}H-thymidine was then added for the last 18 h of culture. Results are expressed as c.p.m. × 10^3 (S.D.) from 6 independent experiments. Mabs were used at 5 μg/10^6 cells concentration; rIL2 at 30 U/ml.
ND = not determined.

Table 2. Proliferative response of TILs and PBLs from 6 patients with lung adenocarcinoma

		^{3}H-thymidine uptake			
Cells	Stimuli	24 h	48 h	72 h	96 h
TIL	None	1.0 (0.2)	1.7 (0.5)	2.1 (1.3)	2.0 (1.3)
	anti-CD28	2.2 (1.4)	8.9 (3.3)	22.8 (8.7)	14.6 (5.0)
	anti-CD2	2.1 (0.9)	3.0 (1.5)	8.7 (1.9)	3.8 (1.1)
	anti-CD3	1.9 (0.2)	2.1 (0.6)	1.7 (1.4)	2.3 (1.0)
	anti-CD2+ anti-CD28	2.7 (0.8)	11.4 (2.0)	21.3 (6.1)	13.0 (4.1)
	rIL2	ND	ND	2.6 (0.7)	2.4 (0.6)
Feeder cells alone		0.8 (0.1)	1.9 (0.7)	0.9 (0.4)	1.4 (0.6)
PBL	None	1.1 (0.6)	2.0 (1.1)	2.4 (0.8)	1.9 (1.2)
	anti-CD28	2.2 (2.0)	6.9 (3.1)	19.2 (3.0)	41.4 (2.6)
	anti-CD2	3.6 (1.7)	7.2 (2.8)	29.0 (6.1)	44.8 (3.8)
	anti-CD3	27.8 (3.2)	109.1 (14.0)	97.8 (4.9)	14.0 (5.0)
	anti-CD2+ anti-CD28	5.6 (2.0)	39.9 (5.1)	72.7 (7.8)	110.1 (6.5)
	rIL2	ND	ND	9.6 (2.0)	10.9 (3.4)

Freshly isolated TILs and PBLs were stimulated for the indicated periods of time with the various Mabs. ^{3}H-thymidine was then added for the last 18 h of culture. Results are expressed as c.p.m. × 10^3 (S.D.) from 6 independent experiments. Mabs were used at 5 μg/10^6 cells concentration; rIL2 at 30 U/ml.
ND = not determined.

Table 3. Proliferative response of fractionated CD45RO$^-$ and CD45RA$^-$ TIL to activation via CD2 or CD28

	^{3}H-thymidine uptake								
	Unfractionated			CD45RA$^-$			CD45RO$^-$		
Stimuli	48 h	72 h	96 h	48 h	72 h	96 h	48 h	72 h	96 h
None	1.8	0.9	1.8	1.6	2.9	2.1	1.4	3.1	0.7
anti-CD2	8.6	17.4	8.9	6.5	8.8	7.2	11.9	13.8	7.6
anti-CD28	21.1	28.6	19.4	25.2	36.6	22.4	10.8	9.6	4.8

Unfractionated, CD45RA or CD45RO TILs were stimulated for the indicated periods of time with the anti-CD2 or anti-CD28 Mab (5 μg/10^6 cells). ^{3}H-Thymidine was added for the last 18 h of culture. Results are expressed as c.p.m. × 10^3 (mean of two independent experiments).

Table 4. TILs release TNF-α and IFN-γ upon stimulation with anti-CD2 and/or anti CD28

	Kidney		Lung	
Stimuli	TNF-α (pg/ml)	IFN-γ (U/ml)	TNF-α (pg/ml)	IFN-γ (U/ml)
None	0	0	10	50
anti-CD2	300	300	290	200
anti-CD28	20	280	20	290
anti-CD2 anti-CD28	600	320	290	300

10^5 TILs were stimulated with the corresponding Mab (50 ng) for 48 h. Cell free supernatants were then harvested and TNF-α or IFN-γ quantitated as described in Materials and Methods.

ways of T cell activation was detectable (not shown). The addition of anti-CD3 Mab did not cause calcium mobilisation at a concentration in excess of that required to produce a maximal response in control PBLs (compare Fig. 3f with Fig. 3c). No difference was observed in the response to the various stimuli between TIL populations derived from either CD2- or CD28-activated cells (not shown).

CD28 and CD45R0 association on human TILs

As the large majority of TILs responsive to anti-CD28 were CD45RO$^-$, we analysed whether CD28 and CD45R0 molecules

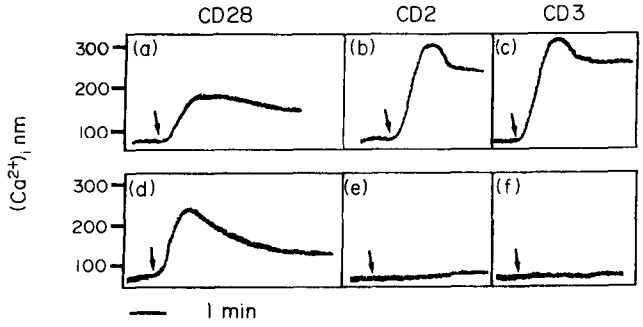

Fig. 3. CD28-induced calcium mobilisation in TILs (d–f) vs. PBL (a–c). Cells were challenged with purified anti-CD28 (d, a) or anti-CD2 (e, b) or anti-CD3 (f, c). Arrows indicate the addition of the corresponding Mabs. Results are referred to one representative experiment out of 3 with TILs and PBLs from renal cell carcinomas patients and 3 from patients with lung tumours.

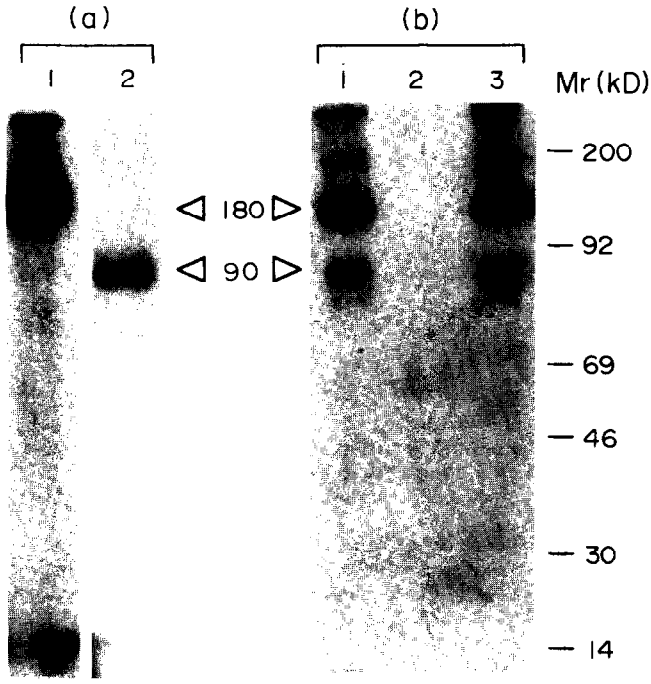

Fig. 4. Co-precipitation of CD28 and CD45RO molecules from activated TILs. TILs were 125I labelled, lysated in lysis buffer with (panel b) or without (panel a) 0.3% CHAPS and immunoprecipitated with anti-CD28 (Kolt2, lanes 2a and 3b) or anti-CD45RO (UCHL1, lanes 1). Samples were run on a 4–12% gradient.

were physically associated at the surface of these cells. It is of note that both anti-CD28 and anti-CD45R0 Mabs co-precipitated two molecules of approximately 180 (CD45R0) and 90 (CD28) kD, respectively (Fig. 4b, lanes 1 and 2). Similar results were obtained with 1% digitonin instead of 0.3% CHAPS lysis buffer (not shown). These findings strongly support the hypothesis that the two molecules are associated on the cell membrane of TILs. The association between CD28 and CD45R0 molecules was further confirmed by solid-phase ELISA (Fig. 5).

DISCUSSION

We demonstrated that: (a) the CD28, but not the CD2 or CD3, pathway of T cell activation is functional in human TILs; (b) fresh TILs are predominantly $CD45R0^+$ and coexpress the CD45RA isoform upon stimulation via CD28 but not via CD2; (c) CD45R0 and CD28 molecules are physically associated at the cell surface of activated TILs. Previous reports indicated that the CD28 molecule could enhance TIL proliferation obtained with anti-CD3 Mab and IL2 [21]. In our experiments TIL could be triggered by anti-CD28 alone, without the need of either anti-CD3 or IL2, thus indicating that CD28 did not merely represent a co-stimulatory signal. As a consequence, it might be hypothesised that the CD28 ligand B7/BB1 is expressed at the tumour site and provides TILs with a stimulatory signal, in keeping with what was observed in another experimental model [22]. On the other hand, primed TILs might undergo clonal expansion and help in the maintenance of local immunity in solid tumours, due to the fact that CD28 stimulation occurs regardless of the presence of a functional CD3 and with a certain delay after TCR–antigen interaction.

The potent effect of CD28 stimulation on TNF-α and lymphotoxin gene expression [23] suggests another potential mechanism by which TILs might exert their antitumour activity. Lymphokine production by lymphocytes co-stimulated with anti-CD3 and anti-CD28 Mabs appears relatively resistant to immunosuppressive drugs, in comparison with cells stimulated through the TCR alone [24, 25]. This could be of interest, as TILs triggered via CD28 might become resistant to the action of immunosuppressive factors produced by tumour cells [26]. The fact that the CD2 activation pathway is functional in inducing TNF-α and IFN-γ production by TIL, but not Ca^{2+} mobilisation and cell proliferation, is intriguing and suggests a functional dissection between proliferation and lymphokine release.

The reason for the preferential responsiveness of TILs to stimuli delivered via CD28 is unclear; with this regard, the CD45 molecule might be involved, as it has been described that stimulation of CD45 with specific Mabs can augment or inhibit T cell activation [12]. In particular, the CD45 molecule has been reported to be essential for coupling the CD3/TCR complex to phosphatidyl inositol pathway [27]. Moreover, in a murine system it has been shown that association of CD45 with the CD3 molecule could allow the more sensitive triggering of peripheral blood T cells [14]. A second clue concerns the association of CD2 and CD45 recently demonstrated, using chemical crosslinking, on human peripheral lymphocytes whose response to anti-CD2 was strongly amplified by anti-CD45 Mabs [13]. Since TILs which did preferentially respond to anti-CD28 were mostly $CD45R0^+$, as demonstrated by sorting experiments, it was tempting to speculate that CD28 associates with CD45R0.

Data from immunoprecipitation and ELISA experiments suggested that preferential association of CD45R0 isoform to CD28 molecule might occur at the surface of human TILs accounting, at least in part, for the preferential activation of $CD45R0^+$ TILs via CD28. Different CD45 isoforms could alternatively associate with different molecules involved in T

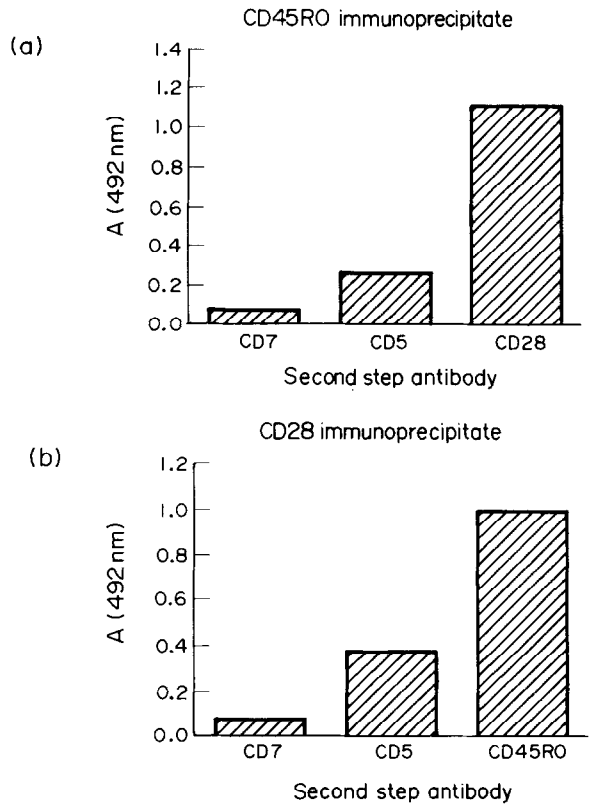

Fig. 5. Association between CD28 and CD45RO molecules on TILs. ELISA was performed on either CD45RO (a) or CD28 (b) immunoprecipitates. Second step antibodies are indicated on the *x*-axis.

cell activation (i.e. CD3, CD2 or CD28), thus regulating signal transduction and TILs function depending on the type of receptor–ligand interaction. Our data suggest that CD28 pathway of T cell activation can play a role in local antitumour immune reactivity, allowing clonal expansion and lymphokine release by primed TIL, independent of the presence of a functional CD3/TCR complex.

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Acknowledgements—We thank Dr G. Chiesa (Surgical Staff, Istituto Scientifico San Raffaele, Milan, I) for providing tumour specimens; Dr D. Olive (INSERM, Marseille, F) for the kind gift of anti-CD2 Mabs; Dr V. Soler for FACS analysis; Dr R. Sitia for helpful discussion. The work was partially supported by a grant from the Italian Association for Cancer Research (AIRC).