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Role of adhesion molecules in lymphokine-activated killer cell killing of bladder cancer cells: further evidence for a third ligand for leucocyte function-associated antigen-1

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SUMMARY

The lysis of eight human bladder cancer cell lines by lymphokine-activated killer cells (LAK) was found to be partially dependent upon the expression by the target cell of either intercellular adhesion molecule-1 (ICAM-1) or intercellular adhesion molecule-2 (ICAM-2). Using adhesion blockade studies these molecules were found to contribute towards sensitivity to lysis. Tumour lines of low grade (G1) did not constitutively express ICAM-1, but were found to express ICAM-2. High grade cells (G2, G3), however, only constitutively expressed ICAM-1 on their cell surface. Interferon-gamma (IFN-γ) induced and augmented the expression of ICAM-1 by all except one of the cell lines (UMUC3) in a dose- and time-dependent manner. This was accompanied by an increased susceptibility to lymphokine-activated killer mediated cytolysis. Anti-ICAM-1 antibodies partially inhibited the increase in cell lysis due to IFN-γ. However, this inhibition was not complete. When effector cells were treated with antibodies to leucocyte function-associated antigen-1 (LFA-1) the inhibition of lysis was greater and ranged from 72 to 96% with a mean of 87% inhibition. These results suggest that the increased sensitivity of IFN-γ-treated bladder cancer cell lines to LAK cells is partially attributable to the induction of ICAM-1. However, blocking of ICAM-1 with antibodies could only partially inhibit the increased LFA-1-dependent lysis. This supports recent evidence for the existence of an additional ligand for LFA-1, other than ICAM-1 and ICAM-2.

INTRODUCTION

Lymphokine-activated killer (LAK) cells are capable of lysing a wide variety of otherwise natural killer (NK)-resistant tumour cells.[1] This cytolytic activity is distinct from T-cell-mediated killing in that it is non-major histocompatibility complex (MHC) restricted and does not affect normal cells.[2] Previously we have demonstrated differential susceptibility to LAK killing of a panel of four bladder cancer cell lines.[3]

The structure(s) involved in non-specific target recognition by LAK effector cells remain largely unknown. However, structures have been identified on the surface of leucocytes which are involved in cell adhesion.[4,5] Prominent among these structures are members of the Leu-CAM (leucocyte cell adhesion molecule) family, CD11a, b, and c/CD18.[6,7]

Abbreviations: ICAM-1, intercellular adhesion molecule-1; ICAM-2, intercellular adhesion molecule-2; IL-2, interleukin-2; IFN-γ interferon-gamma; LFA-1, leucocyte function-associated antigen-1; LAK, lymphokine-activated killer; TCC, transitional cell carcinoma.

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LFA-1 (CD11a/CD18, leucocyte function-associated antigen-1) is the major leucocyte integrin on lymphocytes.[8] There are two characterized ligands for LFA-1, intercellular adhesion molecule-1 (ICAM-1) and ICAM-2 and recent evidence suggests a third counter-receptor may exist.[9,10]

Conflicting evidence exists as to the role of LFA-1 and ICAM-1 in LAK-mediated killing of tumour targets. Quillet-Mary *et al.* demonstrated that target lysis by human LAK cells was dependent upon target binding properties, but LFA-1, LFA-3 and ICAM-1 were not the major adhesion ligands on targets as differences in adhesion molecule expression did not account for the marked differences in susceptibility.[11] In contrast other workers have shown that the susceptibility of colon carcinoma cells to LAK-mediated lysis is associated with elevated expression of ICAM-1 and LFA-3.[12]

Interferon-gamma (IFN-γ) increases the expression of MHC class I, MHC class II, and ICAM-1 by cells of varying origin.[13–15] Studies on cytotoxic T lymphocyte (CTL) antigen-specific lysis of keratinocytes, suggest that both ICAM-1/LFA-1 and Ag/CD3-T-cell receptor (TcR) interactions are important when target cells are stimulated with IFN-γ. In fact, enhanced cytolysis of keratinocytes after short-term treatment with IFN-γ could be explained solely on the basis of ICAM-1 induction.[16]

We have investigated the role of adhesion molecules found to be constitutively expressed by eight transitional cell carcinoma (TCC) cell lines with particular emphasis on their susceptibility to LAK-mediated lysis. Furthermore, we have studied the ability of IFN-γ to modulate antigen expression and examined the functional role of increased adhesion molecule expression. We demonstrate that ICAM-1 and ICAM-2 expression by target cells contributes towards sensitivity to LAK cells and that LFA-1 is the major ligand involved in LAK killing of these targets. Furthermore, the observation of LFA-1-dependent, ICAM-1- and ICAM-2-independent cell interactions adds weight to the existing evidence for a third ligand for LFA-1.

MATERIALS AND METHODS

Cell lines

Eight human transitional cell carcinoma lines RT4, RT112, MGH-U1 (J. Masters, Institute of Urology, London, U.K.), SD, J82, 5637, UMUC3 (P. Perlmann, Stockholm, Sweden) and EJ18 (J. Boyd, University of Edinburgh, U.K.) were used as a tissue culture model of bladder cancer. These cell lines represent histopathological tumour grades 1, 2, 3, 3, 3, 3, 1 and 3 respectively. The cell lines have proved to be stable and their cytological appearances and growth characteristics have remained representative of the parent tumours.

The cell lines were routinely grown in RPMI-1640 (Gibco, Uxbridge, Middlesex, U.K.) supplemented with 5% foetal calf serum (FCS) (Sera-Lab, Crawley Down, Sussex, U.K.), sodium pyruvate (5 mM) and L-glutamine (2 mM). Cells were recovered for routine use in cytotoxicity assays by trypsinization (trypsin/EDTA, 0·5 g/l trypsin and 0·2 g/l disodium EDTA) for 4 min.

Cytokines

Highly purified recombinant IFN-γ was purchased from Boehringer Mannheim (Lewes, U.K.) and contained $2{\cdot}5 \times 10^7$ U/mg of protein and less than 2 endotoxin U/mg. Highly purified recombinant interleukin-2 (IL-2) was generously provided by Euro-Cetus B.V. (Amsterdam, The Netherlands) and contained $1{\cdot}8 \times 10^7$ U/mg of protein.

Monoclonal antibodies

The following murine monoclonal antibodies were generously provided by Dr T. A. Springer (Harvard Medical School, MA); RR1/1 (anti-ICAM-1, IgG1), CBR-IC2/2 (anti-ICAM-2, IgG2a). Anti-LFA-1 (CD11a, IgG1) monoclonal antibodies were obtained from Dako (High Wycombe, Bucks, U.K). Anti-LFA-2 (IgG1) monoclonal antibody was provided by the Scottish Antibody Production Unit (Carluke, U.K.). Anti-HLA-DR (IgG1) antibody was purchased from Dako.

Effector cells

Mononuclear cells were isolated from fresh, heparinized peripheral blood from normal, healthy laboratory volunteers. Briefly, peripheral blood was centrifuged on Ficoll–Isopaque (density = 1·086 g/cm^3) for 30 min at 400 ***g***. The interface was collected and subsequently washed three times in Hanks' Balanced Salt Solution (HBSS) after which adherent cells were removed by adherence to plastic for 1 hr at 37°. The non-adherent cells were collected and washed twice in HBSS.

Generation of LAK cells

Mononuclear cells were cultured at 1×10^6/ml in RPMI-1640 medium supplemented with 5% heat-inactivated FCS and 1000 U/ml recombinant IL-2. The mononuclear cells were incubated at 37° in a humidified atmosphere of 5% CO_2 in air for 6 days. After the incubation the cells were washed twice in HBSS and resuspended at 4×10^6/ml and assessed for cytolytic activity against labelled target cells. The presence of FCS in the medium was not found to influence the generation of LAK cells as compared to serum-free medium (data not shown).

Cytotoxicity assay

Cytotoxicity was measured by means of a 4-hr chromium-release assay. In brief 10^6 target cells were radiolabelled with 100 μCi $Na_2{}^{51}CrO_4$ (Amersham International, Amersham, Bucks, U.K.) at 37° for 30 min with shaking. After multiple washes the targets were resuspended in RPMI-1640 at a density of 5×10^4/ml. Labelled targets (5×10^3/well) were dispensed into the wells of 96-well U-bottomed microtitre plates (Sterilin Ltd, Feltham, U.K.) and then graded numbers of effector cells were added to give effector-to-target ratios (E:T) ranging from 40:1 to 5:1 in a total of 200 μl of medium. After a 4-hr incubation at 37° and centrifugation at 100 ***g*** for 5 min, 100 μl of supernatant was removed from each well and the radioactive content determined using a liquid scintillation counter. All assays were performed in triplicate and the data expressed as the percentage specific cytotoxicity, calculated as below:

$$\% \text{ specific cytotoxicity} = \frac{\text{c.p.m. experimentation release} - \text{c.p.m. spontaneous release}}{\text{c.p.m. total release} - \text{c.p.m. spontaneous release}}.$$

Quantitation of surface antigen expression

Single-colour immunofluorescence was performed on bladder cancer cell lines stained indirectly with monoclonal antibodies and detected with a monoclonal antibody conjugated to fluorescein isothiocyanate (FITC). For each analysis 5×10^5 cells were washed with washing buffer [phosphate-buffered saline (PBS) 1% FCS, 0·01% sodium azide] and incubated at 4° for 30 min with optimal concentrations of primary monoclonal antibody. Following two washes, the binding of unconjugated monoclonal antibodies was detected using a goat anti-mouse-FITC conjugate for a further 30 min at 4°. The cells were washed twice more and resuspended in 1% formaldehyde prior to analysis using a flow micro-fluorometer (Coulter Electronics, Hialeah, FL). Non-viable cells were gated out of the window and at least 10,000 events were accumulated using logarithmic amplification of fluorescence intensity.

Antibody blocking studies

The above cytotoxic assay protocol was modified as follows: ^{51}Cr-labelled target cells or effector cells were incubated with or without monoclonal antibodies (10 μg/ml) in a final volume of 100 μl for 30 min at room temperature. After two washes, target cells were added to each well. LAK cells were also incubated with or without monoclonal antibodies in a similar manner to targets. In some experiments monoclonal antibodies were present throughout the cytotoxicity assay at a concentration of 10 μg/ml IgG.

Table 1. The constitutive expression of ICAM-1 and ICAM-2 by eight bladder cancer cell lines. The percentage of cells expressing each molecule was determined by flow cytometry as detailed in Materials and Methods. The standard deviation of the results was not greater than 10%

Cell line	RT4	UMUC3	RT112	MGH-U1	EJ18	J82	SD	5637
Grade	G1	G1	G2	G3	G3	G3	G3	G3
% cells ICAM-1$^+$	0	0	5	17	72	63	30	50
% cells ICAM-2$^+$	6	33	0	0	0	0	0	0

Table 2. Summary of the sensitivity of eight bladder cancer cell lines to LAK-mediated cytolysis. Results expressed as per cent specific cytotoxicity at a standard effector to target ratio of 40:1. Each value represents the mean of at least five experiments, each performed in triplicate. The standard deviations were within 10%

Cell line	RT4	UMUC3	RT112	MGH-U1	EJ18	J82	SD	5637
Grade	G1	G1	G2	G3	G3	G3	G3	G3
Specific cytotoxicity	40	76	24	40	65	80	50	61

Cytokine treatment of targets

Monolayers of each cell line were grown in 24- or 6-well plates. The cells were cultured in the presence of recombinant IFN-γ at different concentrations (1–2000 U/ml) for 24 hr (unless otherwise stated). Cells were trypsinized and harvested for further analysis.

Statistical analysis

Statistical analysis was performed using the non-parametric Mann–Whitney *U*-test provided in Statview 512 software and using a Macintosh IICX computer.

RESULTS

Constitutive expression of ICAM-1 and ICAM-2 on TCC cells

Using flow cytometry the cell surface phenotype of the eight TCC cell lines was studied. The results are summarized in Table 1. Only two of the eight cell lines failed to express any ICAM-1 (RT4, UMUC3). These two cell lines, both of which corresponded to tumour grade 1, did however express ICAM-2. The G2 cell line, RT112, expressed a small percentage of ICAM-1 but no ICAM-2. The remaining five cell lines, all of which were G3, expressed higher levels of ICAM-1 (17–72% of cells positive) than the G2 and G1 cell lines and failed to express any ICAM-2. None of the cell lines expressed LFA-1, LFA-2 or neural cell adhesion molecule (NCAM) (data not shown).

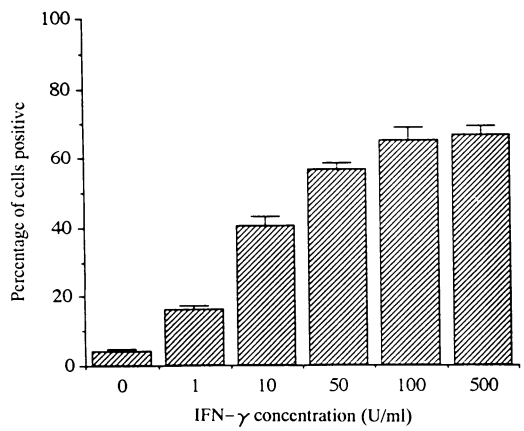

Figure 1. The effect of IFN-γ concentration on the expression of ICAM-1 by RT4. Cells were cultured with increasing concentrations of IFN-γ for 24 hr and ICAM-1 expression determined by flow cytometry.

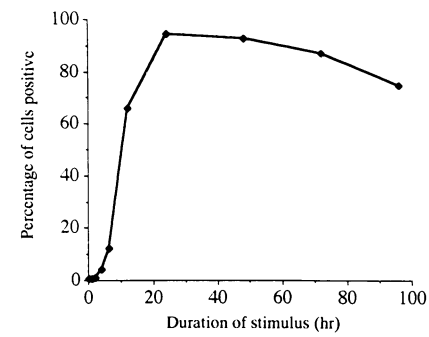

Figure 2. The time-course of induction of *de novo* ICAM-1 expression by RT4. Cells were cultured for the indicated times with 100 U/ml of IFN-γ. ICAM-1 expression was determined using flow cytometry. The kinetics were similar for all lines which responded to IFN-γ treatment. All standard deviations were within 5%.

Susceptibility of TCC cells to LAK killing

We have previously reported the differential susceptibility of four TCC cell lines (RT4, RT112, MGH-U1 and EJ18) to LAK cell killing.[3] This study extends the earlier observations by using an additional four cell lines. The specific cytotoxicity observed against all eight cell lines is summarized in Table 2. There was no

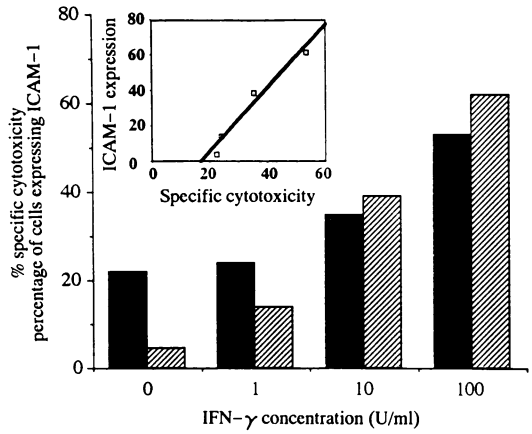

Figure 3. The relationship between ICAM-1 induction and LAK susceptibility in the RT112 cell line. Increased target cell lysis by LAK cells parallels IFN-γ-induced increases in cell surface ICAM-1 expression. Main figure shows the effects of increasing IFN-γ concentration on both ICAM-1 expression (▨) and specific cytotoxicity (■) of RT112 cells by LAK cells. Inset shows correlation between ICAM-1 expression and specific cytotoxicity.

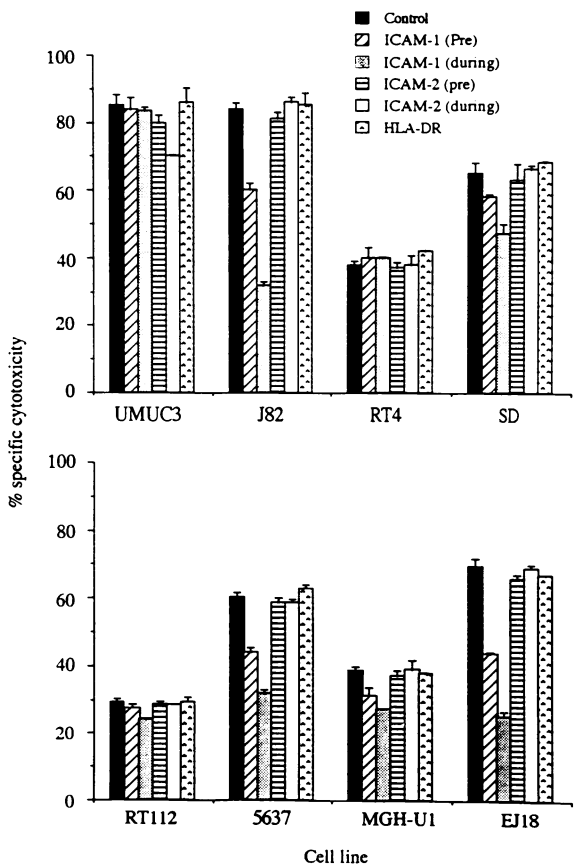

Figure 4. Effects of anti-ICAM-1, anti-ICAM-2 and anti-HLA-DR antibodies on the lysis of untreated TCC target cells by LAK cells. Target cells were incubated with antibodies (10 μg/ml) either prior to (Pre) or during the assay period. Bars represent the mean specific lysis for triplicate assays. Error bars show standard deviation.

apparent correlation between susceptibility to LAK cell killing and grade of the parent tumour.

Modulation of adhesion molecule expression by IFN-γ

We analysed the effect of IFN-γ on the expression of ICAM-1 and ICAM-2 molecules by TCC cell lines. IFN-γ induced or augmented the expression of ICAM-1 molecules on all cell lines except UMUC3. ICAM-1 expression could not be induced on UMUC3 even by stimulation with massive amounts of IFN-γ (2000 U/ml) for prolonged periods of time (200 hr) (data not shown). The sensitive cell lines, however, responded to as little as 1 U/ml IFN-γ and maximum induction was achieved with 100 U/ml (Fig. 1). Representative kinetics of induction are shown in Fig. 2 indicating ICAM-1 expression was maximal after 24 hr stimulation.

The expression of ICAM-2 on TCC cell lines could not be augmented or induced by stimulation with IFN-γ (data not shown). Furthermore, LFA-1, LFA-2 and NCAM expression were not induced on any of the cell lines following treatment with IFN-γ (data not shown).

Increased susceptibility of IFN-γ-treated TCC cell lines to lysis by LAK cells

TCC cells were stimulated with different concentrations of IFN-γ for 24 hr and their sensitivity to lysis by LAK cells was examined. After treatment with as little as 10 U/ml IFN-γ a significant ($P<0.01$) increase in specific cytotoxicity was observed. Treatment with 100 U/ml resulted in a further increase in specific cytotoxicity ($P<0.001$). Although 1 U/ml caused an increase in ICAM-1 expression no significant increase in specific cytotoxicity was observed. Figure 3 shows the specific cytotoxicity obtained against RT112 target cells treated with IFN-γ for 24 hr and the percentage of cells expressing ICAM-1. Correlation between induced ICAM-1 expression on RT112 and LAK killing was evident (Fig. 3, inset). An increase in susceptibility after treatment with IFN-γ was noted for all lines including UMUC3, but no increase in ICAM-1 expression was noted for this line. Previously we have reported the cytotoxic and cytostatic effects of IFN-γ against bladder cancer cells; there was, however, no significant difference in spontaneous release of ^{51}Cr by untreated or treated cells.[17]

Blocking of adhesion molecules on target cells with antibodies decreases sensitivity to LAK killing

The functional roles of constitutive ICAM-1 and ICAM-2 expression were analysed by monitoring the effects of antibodies to these structures on the lysis of TCC cell lines. Figure 4 shows representative blocking studies for all cell lines. Monoclonal antibody blocking was performed prior to the assay or during the assay. Specific cytotoxicity against J82, MGH-U1, EJ18, 5637 and SD was decreased by blocking with anti-ICAM-1 antibody. This effect was more marked if the antibody was present throughout the assay period. Anti-ICAM-2 antibodies had no effect on sensitivity of these five lines.

LAK killing of UMUC3 (one of the two ICAM-2$^+$ cell lines) was decreased only when anti-ICAM-2 antibodies were present during the assay. The sensitivity of RT4 and RT112 could not be altered by attempted blocking with antibody either prior to or

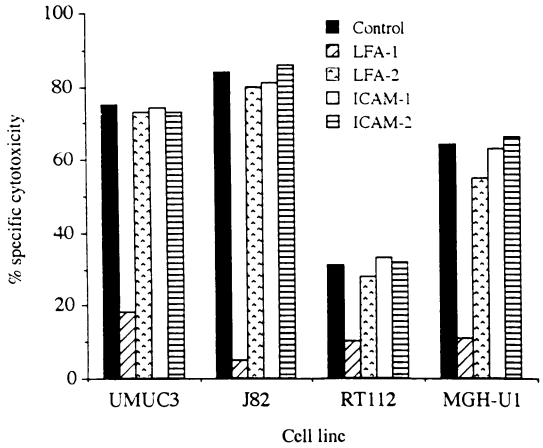

Figure 5. Effects of anti-LFA-1, anti-LFA-2, anti-ICAM-1 and anti-ICAM-2 antibodies on the ability of LAK cells to kill four TCC targets. Effector cells were incubated with antibodies (10 μg/ml) for 30 min at room temperature prior to assay. Control cells were incubated as above in the absence of antibodies. Standard deviations were within 10%.

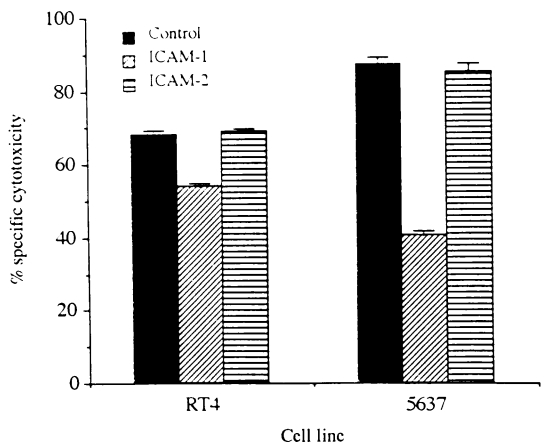

Figure 6. Effect of blocking IFN-γ-treated target cells using antibodies to ICAM-1 or ICAM-2. Control represents IFN-γ-treated targets which were not blocked with antibodies. Error bars show standard deviation.

during the assay period. Monoclonal antibodies to HLA-DR did not modify the sensitivity of any of the lines.

Although antibodies to adhesion molecules ICAM-1 and ICAM-2 were seen to modify the sensitivity of TCC targets to LAK killing, the killing could not be completely blocked, even in the presence of increased antibody concentration.

Blocking of LFA-1 on LAK cells with antibodies decreases their potential for target killing

To examine further the role of adhesion molecules in LAK killing of bladder cancer targets, we studied the effect of blocking adhesion structures present on the effector cells. Figure 5 details the effects of blocking LAK cells on their ability to lyse four TCC targets. When effector cells were treated with monoclonal antibodies to LFA-2, ICAM-1 or ICAM-2, no change was observed in LAK potential against any of the four targets. However, when LAK cells were coated with anti-LFA-1 antibodies, a marked and significant decrease ($P < 0.001$) in specific cytotoxicity was seen. The percentage decrease ranged from 72% (RT112) to 96% (J82) with a mean of 84% for all eight cell lines.

Blocking of induced adhesion molecules expressed on target cells

When IFN-γ-treated targets were blocked with antibodies to ICAM-1 their susceptibility was decreased. However, the decrease was not as marked as observed in uninduced cells which had also been blocked using antibodies (Fig. 6).

DISCUSSION

We have examined the constitutive expression of adhesion molecules on the surface of eight bladder cancer cell lines and studied their susceptibility to LAK-mediated lysis. We have also studied the effect of IFN-γ treatment on adhesion molecule expression and susceptibility to LAK killing. Our results show that all eight cell lines constitutively express either ICAM-1 or ICAM-2 but not both. Furthermore, the constitutive expression of ICAM-2 appears to be restricted to G1 tumour lines and ICAM-1 expression limited to G2 and G3 cells. When TCC cells were treated with IFN-γ, ICAM-1 expression was induced or augmented on seven out of eight cell lines. In contrast, ICAM-2 expression remained unchanged. Treatment with IFN-γ resulted in increased sensitivity to LAK-mediated lysis. This was shown not to be an artefact due to the known cytotoxic/cytostatic effects that IFN-γ has on TCC cell lines.[17] Anti-ICAM-1 or ICAM-2 antibody partially inhibited both basal LAK sensitivity and augmented sensitivity of the targets. However, treatment of effector cells with anti-LFA-1 antibody, more effectively inhibited the LAK killing than ICAM-1 or ICAM-2 antibodies.

Whilst the blocking of ICAM-1 or ICAM-2 expressed by TCC targets decreased the LAK susceptibility, blocking of these molecules on effector cells had no effect (see Fig. 5), thus demonstrating that antibody was not shed during the LAK assay, as blocking of target structures would reduce the killing. Furthermore, the target cells did not express the ligand for ICAM-1 and ICAM-2. The observation that antibody against target cell ICAM-1 did not inhibit cytotoxicity as effectively as antibody against effector cell LFA-1 is unlikely to be as a result of the participation of ADCC as the anti-DR antibody did not enhance cytotoxicity. The use of F(ab')$_2$ fragments of anti-ICAM-1 antibody might have confirmed this.

The increased susceptibility of IFN-γ-treated target cells to LAK-mediated killing is partially due to the increased expression of ICAM-1 as shown by blockade studies using anti-ICAM-1 antibodies. Although IFN-γ also induces the expression of HLA class II molecules on the surface of the TCC cell lines[13] these molecules are not thought to be involved for two reasons: Firstly, antibodies to HLA class II molecules failed to alter the sensitivity of the targets to LAK killing. Secondly, the kinetics of HLA class II induction is markedly different from those of ICAM-1 induction, being slower and less sensitive to IFN-γ.[13] One further explanation is that the increased susceptibility of IFN-γ-treated TCC cells to LAK-mediated lysis is attributable, in addition to the increased expression of ICAM-1, to a mechanism distinct from effector-target binding.

In marked contrast to our present studies, previous reports have demonstrated that IFN-γ treatment of tumour cells decreased the susceptibility to LAK-mediated lysis.[18] It has been suggested that protection from LAK- and NK-mediated lysis of target cells occurred in the post-binding stage, preventing triggering and activation of the LAK cell[19] or programming for lysis or secretion of factors.[20] This is clearly not the case for IFN-γ-treated bladder cancer cell lines.

The blocking of LFA-1 on effector cells caused almost complete abrogation of basal TCC cell killing and induced killing in most cell lines. However, blocking of ICAM-1 (ligand for LFA-1) on the target cells failed to result in the same degree of inhibition of LAK killing. These LFA-1-dependent, ICAM-1-independent phenomena could not be accounted for by the presence of ICAM-2 on the target cell. These findings extend those of Springer by providing more evidence for the existence of a third counter-receptor for LFA-1.[10] Further evidence is provided by the observation that blockade of induced targets failed to result in killing levels as low as achieved by blockade of uninduced targets; however, blockade of LFA-1 on effector cells resulted in levels equivalent to those achieved against uninduced targets. It is possible, however, that other post-cell binding events were modified by treatment with IFN-γ and these events may play a role in increased killing. Future studies aim to evaluate the role of post-binding events in LAK-mediated cytotoxicity.

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