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Marking hypoxic cells for complement and cytotoxic T lymphocyte-mediated lysis: using pimonidazole

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Summary Artificial antigens are created when 2-nitroimidazoles bind to hypoxic cells. These antigens have been used in the immunodetection of tumour hypoxia but they might also serve to stimulate immune lysis of hypoxic tumour cells by complement- and cell-mediated processes. In order to test this hypothesis, lymphocytes isolated from the spleens of C3H/HeN mice that had been immunised with pimonidazole-labelled 3152-PRO cells were subcultured and tested for their ability to lyse chromium-51 loaded, pimonidazole-labelled 3152-PRO cells in an *in vitro* assay. In a parallel study, commercially available, rabbit complement was tested for its ability to lyse pimonidazole-labelled V79-4 cells in the presence of monoclonal antibodies which recognise protein adducts of reductively activated pimonidazole. Complement-mediated cell lysis was measured by means of an MTT assay. Complement-mediated and cell-mediated lysis was observed at pimonidazole concentrations which, in themselves, do not produce cell killing.

Keywords: hypoxia marker; nitroimidazole; artificial antigen

Artificial, tumour-associated antigens formed when hypoxia markers bind to hypoxic tumour cells have been used diagnostically but the antigens might also serve to target hypoxic tumour cells for immunotherapy. Unlike naturally occurring, tumour-associated antigens such as those derived from oncogenes or mutated tumour suppressor genes (Melief and Kast, 1993), hypoxia marker antigens can be created at will and, to a large extent, independently of cell genetics. While marker-based immunotherapy would require that antibodies and/or cytotoxic T lymphocytes reach hypoxic cells 150 to 200 μm from blood vessels, both the existence of antibodies distant to vasculature (e.g., Baxter *et al.*, 1992) and the presence of lymphocytes within tumours is well known (e.g. Mulé and Rosenberg, 1990). In one case, lymphocytes have been observed in close proximity to tumour cells labelled with hypoxia markers (Cline *et al.*, 1990). It is also required that normal tissues labelled with the markers be protected. Strategies to limit normal tissue labelling are, in principle, available and it was of interest to discover if the primary requirement for success exists; that is, that targeted cells are lysed by complement and/or cytotoxic T lymphocytes (CTLs).

Any compound that can be reductively activated and bound to cellular macromolecules in an oxygen-dependent manner could be used to target hypoxic cells for immune lysis but we chose to investigate compounds such as pimonidazole for which monoclonal antibodies are available from hypoxia marker studies (Arteel *et al.*, 1995). Hypoxia marker antigen concentrations of ca 0.03 mM have been measured in solid tumours (Thrall *et al.*, 1994) but it is not known what percentage of the binding occurs on the surface of cells. However, assuming that tumour tissue contains 10^8 to 10^9 cells g^{-1}, only 1% of the binding need be on the surface of cells to generate the 10^5 to 10^6 antigens required for efficient complement-mediated lysis (Fogler *et al.*, 1989).

In the drug discovery phase of our study, complement-mediated lysis of pimonidazole-labelled V79-4 cells was examined *in vitro* and cell-mediated lysis was investigated in an *in vitro* model based on 3152-PRO tumour cells. The 3152-PRO tumour is a model derived originally as an epithelial cell tumour induced in ultraviolet-irradiated C3H/HeN mice by the subcutaneous injection of 3-methylcholanthrene under ventral, non-UV exposed skin. This model has been used previously for hapten-dependent, tumour rejection studies (Flood *et al.*, 1987).

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Materials and methods

Racemic pimonidazole hydrochloride was synthesised according to published procedures (Smithen and Hardy, 1982) and fully characterised by elemental, spectroscopic and chromatographic analyses. Chromium-51 was obtained as the sodium chromate salt from Amersham Corp. (Arlington Heights, IL, USA); Nonidet P-40, Sigmacote, rabbit complement, mitomycin C and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma Chemical (St. Louis, MO, USA); recombinant cytokines from Genzyme Corp (Cambridge, MA); and other chemicals, buffers and tissue culture media from local suppliers. Mouse monoclonal antibodies (Mabs) to protein-bound pimonidazole were prepared by the North Carolina State University Hybridoma Facility (Raleigh, NC, USA) and purified by affinity chromatography by means of an Immunopure IgG purification kit from Pierce Chemical (Rockford, IL, USA) and appropriate elution buffers for each Mab isotype. Isotyping was carried out with a Clonotyping System/AP kit purchased from Fisher Scientific (Pittsburgh, PA, USA).

In order to label cells with pimonidazole antigens, cells were suspended at $3-5 \times 10^5$ cells ml^{-1} in Eagle's minimum essential medium (MEM) (V79-4 cell experiments) or Roswell Park Memorial Institute (RPMI-1640) medium (3152 PRO cell experiments) at pH 7.4. Both media contained 10% fetal bovine serum (FBS). The cell suspensions were placed in silanised (Sigmacote) 125 ml glass gas collection tubes (Ace Glass, Vineland, NJ, USA) that had been mounted on the deck of an orbital shaker (Model SS110504, Integrated Separation Systems, Natick, MA, USA). The cell suspensions were equilibrated with 5% CO_2 in nitrogen by means of 15 gas exchanges under partial vacuum over a period of 10 min. Cell viability measured by trypan blue exclusion or MTT assay was unaffected by this physical treatment. The hypoxic cells were incubated in the presence of 0 (control), 100, 200, 300, 400, 500 or, in the case of cells for CTL stimulation, 1000 μM pimonidazole for 2 h with agitation under a continuous flow of 5% CO_2 in nitrogen at 37°C. Cells were harvested and washed with phosphate-buffered saline (PBS) to remove unbound pimonidazole. The amount of pimonidazole bound to the cells was analysed by enzyme-

linked immunosorbent assay (ELISA; Arteel *et al.*, 1995; Raleigh *et al.*, 1994). Protein content of the cell suspensions was measured by a modified Lowry technique (Bio-Rad DC protein assay; Bio-Rad Labs, Melville, NY, USA).

In a typical complement lysis study, pimonidazole-labelled V-79-4 cells were washed and incubated in PBS with purified IgG_1 or IgG_3 monoclonal antibodies for 1 h at 4°C. Washed cells were then mixed with rabbit complement (final dilution of 1 : 20), distributed in a 96-well microtitre plate (2 or 4×10^4 cells per well per 100 μl) in Eagle's MEM containing 10% FBS and incubated overnight at 37°C in a 5% CO_2 incubator to allow for cell attachment of surviving cells. A 100 μl solution of 1.5 mg ml^{-1} of MTT in PBS was added and the plates held at 37°C for 4 h. Supernatant fluid was gently poured off and 200 μl per well of dimethyl sulphoxide added and mixed well to solubilise formazan crystals. Absorbance at 490 nm was assayed in a microplate reader (Molecular Devices, Palo Alto, CA, USA) and corrected for absorbance due to medium and MTT alone. Cell survival was recorded as the ratio of absorbance in the absence and presence of complement. The MTT assay has been shown to provide results similar to those for clonogenic assays (Carmichael *et al.*, 1987) and was considered adequate for the present studies.

In a typical CTL study, C3H/HeN mice were immunised by repeated, subcutaneous injections of 3×10^5 3152 PRO cells prelabelled under hypoxia with 200 μM pimonidazole as described above. Twenty one days after the last injection, splenic lymphocytes were isolated and spun through lymphocyte separation medium to remove red blood cells and placed into culture at a dilution of 5×10^6 cells per well in a 24-well tissue culture plate. The CTL experiment involved two steps. In the first step, 3152 PRO cells that had been labelled under hypoxia in the presence of 0 (control), 200, 500 or 1000 μM pimonidazole were added on day 1 to the lymphocyte effector cells. (In all experiments, control and pimonidazole-labelled 3152 PRO cells were incubated with 100 μg mitomycin C for 1 h at 37°C before being added to the lymphocytes in order to inhibit proliferation of the tumour cells). The object of the first step in the experiment was to determine if pimonidazole-labelled cells could stimulate CTL activity against pimonidazole antigens. In some experiments, recombinant interleukins IL-1α, IL-2, IL-4 and interferon gamma (IFNγ) in amounts of 100, 100, 1000 and 100 Biological Response Modifiers Program (BRMP) units respectively were added to the lymphocyte cultures on day 1 to determine if cytokines could further enhance the generation of CTL activity. The effector lymphocyte cells were incubated in the presence of the various additives at 37°C for 6 days. In the second step of the experiment, cytotoxic activity in the lymphocyte cultures against control or pimonidazole-labelled 3152 PRO cells was measured in a ^{51}Cr-release assay according to procedures described previously (Horvat *et al.*, 1991). Briefly, 10^6 unlabelled control or pimonidazole-labelled 3152 PRO tumour cells were labelled with 100 μCi of ^{51}Cr-sodium chromate for 1 h at 37°C. Samples (100 μl per well) of lymphocyte effector cells were placed in U-bottom 96-well microtitre plates (Linbro, Flow Laboratories, McLean, VA, USA) and 10000 ^{51}Cr-labelled target cells added to create a final volume of 200 μl per well of complete medium. The relative proportions of effector and target cells were adjusted to give effector/target cell ratios of 25, 50, 75 and 100 to 1. The mixtures were incubated for 4 h at 37°C. Maximal ^{51}Cr release was defined by incubating target cells in 1% v/v of the non-ionic detergent, Nonidet P-40. Spontaneous release was determined by incubating target cells in medium and was always <20% of maximal release. The percentage of cytotoxicity was determined as follows:

$$\% \text{ Cytotoxicity} = [\text{experimental} - \text{spontaneous}/\text{total} - \text{spontaneous}] \times 100$$

Results

Complement-induced lysis of pimonidazole-labelled V79-4 cells occurred *in vitro* in the presence of anti-pimonidazole antibodies (Figure 1). The extent of lysis increased as the amount of pimonidazole bound to the cells increased (compare Figure 2) but appeared to become significant only at antigen densities above 70 nmol g^{-1} of protein in the cells; that is, for hypoxic target cells that had been incubated in the presence of ⩾ 200 μM pimonidazole. Importantly, cell survival in the absence of complement was high (93±4%) and essentially independent of pimonidazole concentration in the range 100–500 μM so that little or none of the cell lysis recorded in Figure 1 was due to direct pimonidazole toxicity. Data in Figure 1 are for complement lysis in the presence of anti-pimonidazole IgG_3 but an IgG_1 antibody gave similar results under the conditions of Figure 1 (20 μg ml^{-1} antibody). IgG_3 was more effective than IgG_1 at lower Mab concentrations (0.1 to 15 μg ml^{-1}; data not shown).

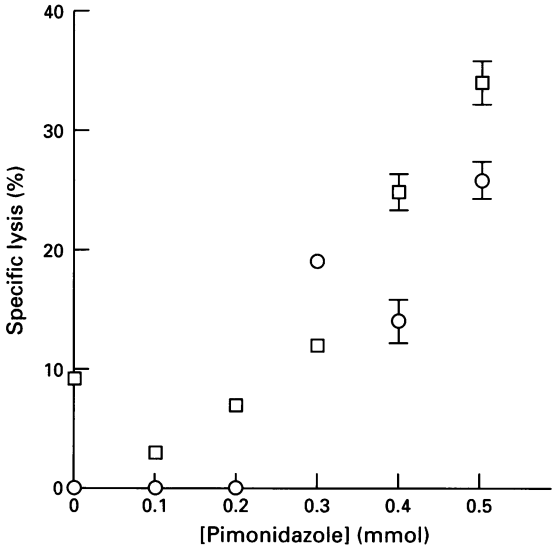

Figure 1 Complement-mediated lysis of V79-4 cells labelled under hypoxia in the presence of increasing concentrations of pimonidazole. Two different cell densities were studied in the MTT assay: (○) 40 000 cells per well and (□) 20 000 cells per well. The results are from a representative experiment in which MTT assays were performed in triplicate for each pimonidazole concentration.

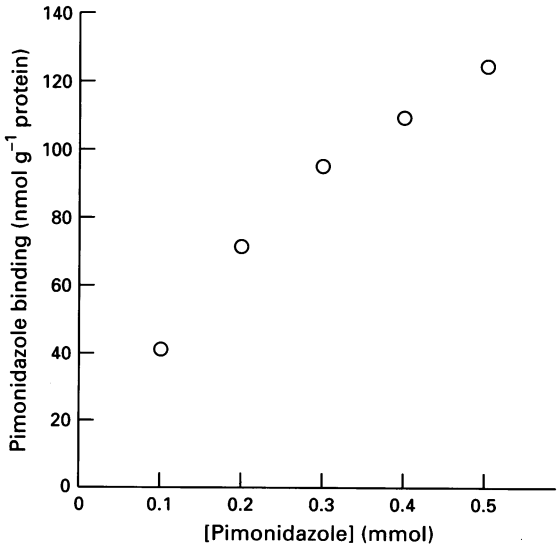

Figure 2 ELISA of pimonidazole binding in V79-4 cells incubated for 2 h under hypoxia as a function of pimonidazole concentration in the cell suspension. The intensity of pimonidazole binding is normalised to cell protein content.

CTL lysis of 3152 PRO cells that had been labelled for 2 h under hypoxia in the presence of 200 μM pimonidazole was observed (Figures 3). As with complement-mediated lysis, CTL induced lysis was easily observable for target cells labelled under hypoxia in the presence of pimonidazole at ⩾ 200 μM.

The splenic lymphocytes isolated from immunised mice had to be stimulated with pimonidazole-labelled cells before they showed full CTL activity toward pimonidazole-labelled target cells (Figure 3a). Stimulatory cells labelled in the presence of 500 μM pimonidazole were used in the first step of the experiments which generated the data in Figure 3 but cells labelled in the presence of 200 or 1000 μM pimonidazole produced a similar stimulatory effect (not shown). A further, small and statistically insignificant, increase in CTL effectiveness could be obtained if the CTL effector cells were coincubated with pimonidazole-labelled cells and 100 BRMP units of the recombinant interleukin, rIL-1α, in the first step of the experiment (compare Figure 3a and b). None of the other cytokines investigated produced this effect.

A significant degree of lysis occurred for 3152 PRO target cells that were not labelled with pimonidazole (Figure 4). The lysis of unlabelled cells did not occur when 3152 PRO cells unlabelled with pimonidazole were used to immunise C3H/HeN mice. As was the case for pimonidazole-labelled target cells, lysis of unlabelled target cells was stimulated by preincubation of the CTLs with pimonidazole-labelled cells and a further small, statistically insignificant, stimulatory effect of IL-1α was seen (compare Figure 4a and b).

Discussion

Immunohistochemical analysis indicates that the binding of reductively activated hypoxia markers is predominantly

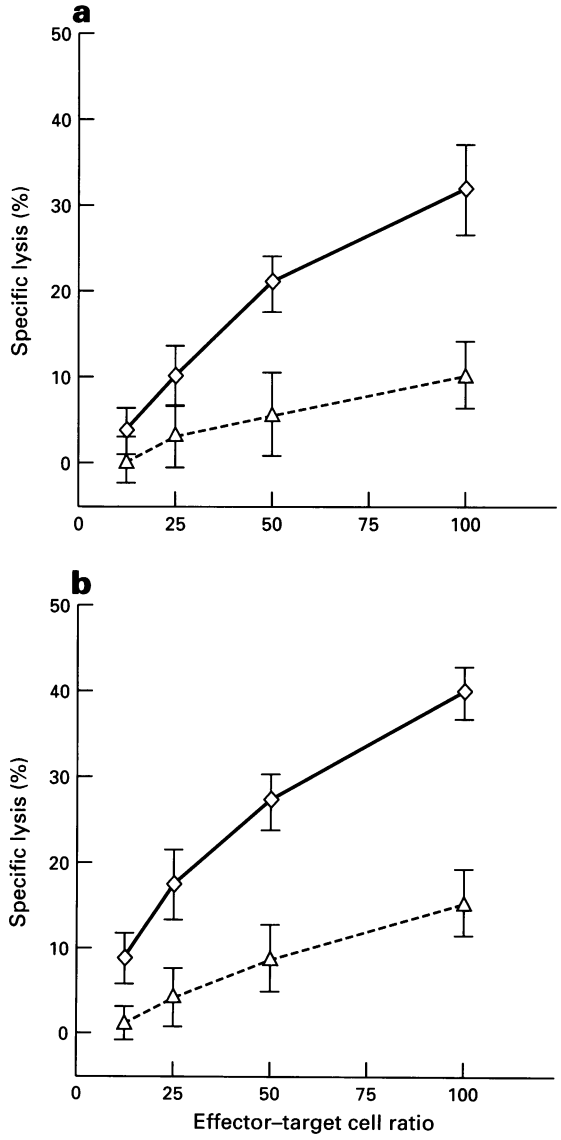

Figure 3 Cytotoxic T lymphocyte-mediated lysis of pimonidazole-labelled 3152-PRO target cells. CTL effector cells were generated *in vitro* from spleen lymphocytes isolated from C3H/NeH mice that had been immunised with pimonidazole-labelled 3152-PRO cells. Before being tested for their ability to lyse pimonidazole-labelled target cells, the lymphocytes were incubated for 6 days with 3152-PRO cells that had been labelled under hypoxia with 0 (△) or 500 (◇) μM pimonidazole. The CTLs were generated in the absence (**a**) or in the presence (**b**) of 100 BRMP units of rIL-1α.

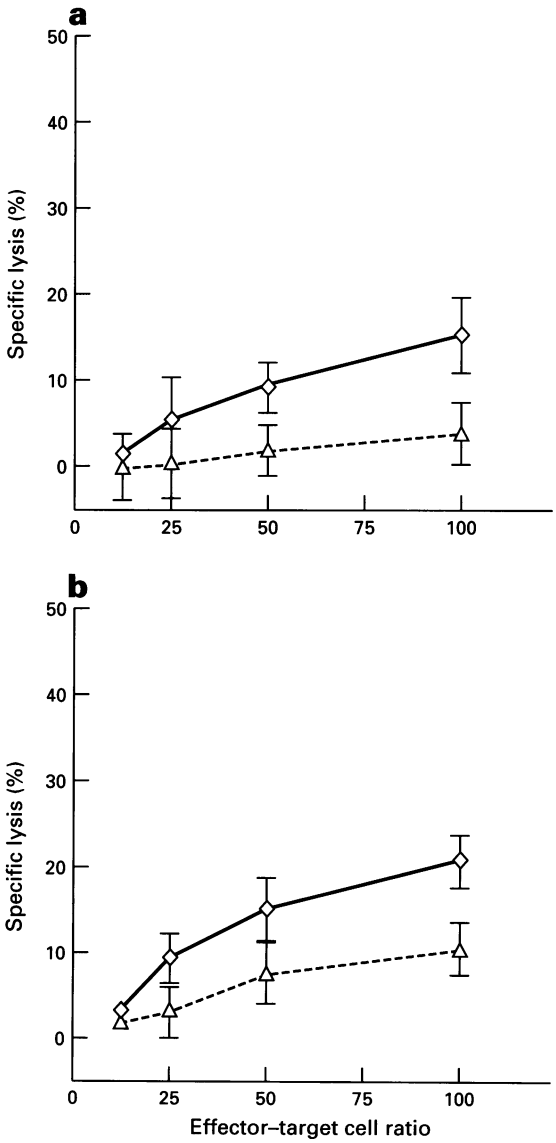

Figure 4 Cytotoxic T lymphocyte-mediated lysis of control, unlabelled 3152-PRO target cells. CTL effector cells were generated *in vitro* from splenic lymphocytes isolated from C3H/NeH mice that had been immunised with pimonidazole-labelled 3152-PRO cells. Before being tested for their ability to lyse unlabelled target cells, the lymphocytes were incubated for 6 days with 3152-PRO cells that had been labelled under hypoxia with 0 (△) or 500 (◇) μM pimonidazole. The CTLs were generated in the absence (**a**) or in the presence (**b**) of 100 BRMP units of rIL-1α.

intracellular (Cline *et al.*, 1994) but the present results indicate that there is sufficient pimonidazole binding on or near the surface of cells to support both complement- and cell-mediated lysis *in vitro*. The concentration of pimonidazole in the labelling solution required to elicit complement lysis *in vitro* is relatively high in both cases ($\geqslant$ 200 μM) but prolonged exposure to lower concentrations of pimonidazole, which could mimic *in vivo* exposure, might achieve the same effect.

The exact nature of the antigen bound to the surface of the hypoxic V79-4 cells is not known but the hapten is presumably similar to glutathione adducts (Yates *et al.*, 1995) and could incorporate the thiol moieties of membrane proteins (Raleigh and Koch, 1990). Possible alternatives include binding to unsaturated lipids in membranes (Raleigh *et al.*, 1981). Antigenic haptens could be created by concomitant reductive activation and binding within the cytoplasmic membrane or by cytosolic activation followed by migration and binding of the reactive intermediates to the cytoplasmic membrane. Binding of reductively activated misonidazole to extracellular bovine serum albumin has been reported for hypoxic cultures of EMT-6 and V79 cells to the extent of $\leqslant$3% of total cellular binding (Chapman *et al.*, 1983). This indicates that activated misonidazole intermediates were available at cell surfaces. The complement-mediated lysis of pimonidazole-labelled cells indicates that this is also true for pimonidazole. However, neither result resolves the question of whether activation occurs within the cytoplasmic membrane or within the cytosol. This distinction might be important in optimising complement-or cell-mediated lysis.

Cell-mediated lysis of pimonidazole-labelled cells is more complex than complement-mediated lysis. For example, 3152 PRO cells unlabelled with pimonidazole were lysed by the CTLs but not by complement. CTL lysis of unlabelled cells is specific in the sense that immunisation with unlabelled 3152 PRO cells does not produce lymphocytes that are cytotoxic to either labelled or unlabelled cells. While the basis of this effect on unlabelled cells is not known, immunisation of C3H/HeN mice with pimonidazole-labelled cells might produce a 'bystander' effect whereby strong, pimonidazole-derived antigens elicit a response to weak, cell surface antigens on 3152 PRO cells that in themselves do not elicit a response. A similar effect has been observed with a trinitrophenyl hapten and it is possible that both trinitrophenyl and pimonidazole hapten effects might be analogous to bystander stimulation often seen in studies of highly antigenic tumours (Flood *et al.*, 1987). The basis of the slight, albeit statistically insignificant, effect of rIL-1α on CTL cytotoxicity is intriguing but does not at present provide an insight into the nature of the cellular or cytokine response of cytotoxic T lymphocytes to pimonidazole-labelled tumour cells. Further work is needed to resolve the specificity and cytokine dependency of the cell-mediated immune lysis reported here.

In summary, sufficient artifical antigen concentrations can be produced on hypoxic cells to elicit both complement- and cell-mediated lysis of pimonidazole-labelled hypoxic cells *in vitro*. Optimisation of conditions, including investigations of other hypoxia markers, and extension of the *in vitro* studies to *in vivo* testing appears warranted for both and complement- and CTL-induced lysis.

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