Eur J Cancer, Vol. 26, No. 8, pp. 863–864, 1990.
Printed in Great Britain

0277–5379/90 $3.00 + 0.00

Fax Communication

Natural Cytotoxic Cells and Tumour Surveillance *in vivo*

Y. Cheng Smart, Paul A. Tooney, Margaret L. Farrelly, Joan H. Brien and Robert C. Burton

INTRODUCTION

WE HAVE described a murine monoclonal antibody (Mab) 1C4 that identifies an alloantigen, NC-1.1, on murine natural cytotoxic cells [1, 2]. We report that NC-1.1$^+$ cells mediate *in vivo* surveillance against WEH1-164, a transplantable fibrosarcoma induced by 3-methylcholanthrene in BALB/c mice [3].

MATERIALS AND METHODS

Mice. CBA and (CBA × BALB/c)F_1 mice of both sexes were obtained from the Central Animal House, University of Newcastle, and used at 6–13 weeks of age.

Tissue culture medium. Dulbecco's modification of Eagle's medium (Flow) was supplemented with 2 mmol/l L-glutamine, 20 mmol/l HEPES, 50 μmol/l 2-mercaptoethanol, 50 μg/ml gentamicin and 10% fetal calf serum.

^{51}Cr release assay. Spleen cells were harvested from at least two mice for each experiment and assayed on ^{51}Cr-labelled WEH1-164 and ^{51}Cr-labelled YAC (Maloney-leukaemia virus induced T-lymphoma in A/Sn mice) target cells for 18 h [4]. Results were first calculated as percent specific lysis, and then lytic units (LU) were derived from the linear slope of the graph of percent specific lysis *vs.* effector/target ratio. One LU was defined as the number of effector cells that mediated 20% specific lysis of the target cell, and results were expressed as LU/10^8 spleen cells.

Flow cytometry. CBA spleen cells were reacted with fluorescein isothiocyanate conjugated F(ab')$_2$ fragments of the 1C4 Mab (IgG_1) and analysed on an 'EPICS Profile' (Coulter Electronics) flow cytometer [5].

Splenic natural cytotoxic activity. For *in vitro* studies CBA spleen cells were pre-incubated with pre-titred ascites 1C4 and washed twice, or reacted with 'Dynabeads M450' coated with sheep anti-mouse IgG (Dynal) that had been pre-treated with 1C4 ascites. The cells were then exposed to a magnetic field to remove the NC-1.1$^+$ cells before assay on WEH1-164 and YAC, both labelled with ^{51}Cr. In addition, groups of CBA mice were given intraperitoneal injections of 200 μl (1:5) neat 1C4 ascites. 24 h later the spleen cells were harvested for assay *in vitro* on labelled WEH1-164 and YAC. In all these experiments tissue culture medium or NS-1 ascites were used as the negative control.

Growth of WEH1-164. Groups of (CBA × BALB/c)F_1 mice were given intraperitoneal injections of 200 μl (1:5) 1C4 ascites or tissue culture medium or NS-1 ascites on days −3, 0, 2 and 3. On day 0, 10^6 WEH1-164 cells were subcutaneously injected at a single site. The mice were examined every 2–3 days and two diameters at right angles were measured on the tumours which appeared until necrosis set in, at which time the mice were killed. The mean tumour diameters in control and treated mice were compared at each time point with Student's *t* test.

RESULTS AND DISCUSSION

Figure 1 shows a cytogram and three fluorescent histograms of 250 000 CBA spleen cells reacted with FLTC-IC4 F(ab')$_2$ and analysed by flow cytometry. On the cytogram three clusters of cells can be distinguished on the basis of their forward-scattering and side-scattering characteristics. The fluorescent histograms show the whole cytogram, the two smaller clusters and the smallest cluster alone. Most of the NC-1.1$^+$ cells fell within the smallest cluster, which has the characteristics expected of large granular lymphocytes. Compared with controls, 20–22% of these large granular lymphocytes were NC-1.1$^+$ and consisted of bright (60%) and dim (40%) fluorescing sub-populations. NC-1.1$^+$ cells made up less than 1% of the total CBA splenocytes.

Table 1 shows data from three experiments which demonstrate that, as we have reported [1,2], splenic natural cytotoxic activity against WEH1-164 *in vitro* can be inhibited or abolished by *in vitro* pretreatment of spleen cells with 1C4 alone or 1C4 coupled to Dynabeads, and by a single intraperitoneal injection of 1C4 24 h before spleen cell harvest. In contrast, splenic natural killer activity against YAC *in vitro* was hardly affected by these *in vitro* and *in vivo* 1C4 treatments. These data support the conclusion that most, if not all, cells with natural cytotoxic activity react with 1C4—i.e. they are NC-1.1$^+$.

Results from a representative experiment of four done to examine the effect of 1C4 on the growth of WEH1-164 in (CBA × BALB/c)F_1 mice are shown in Fig. 2. Throughout the 22 day study, the 1C4-treated mice always had a larger mean tumour

Correspondence to R.C. Burton.
The authors are at the Discipline of Surgical Science, Faculty of Medicine, University of Newcastle, N.S.W. 2308, Australia.

Table 1. *Effect of* 1C4 *on CBA splenic natural killer activity against YAC and natural cytotoxicity against WEH*1-164

	In vitro				*In vivo*	
	IC4 alone		1C4–Dynabeads			
Treatment	YAC	WEH 1-164	YAC	WEH 1-164	YAC	WEH 1-164
Control	139*	357	830	200	833	312
1C4	101	0	769	0	800	0

*Mean LU/10^8 spleen cells.

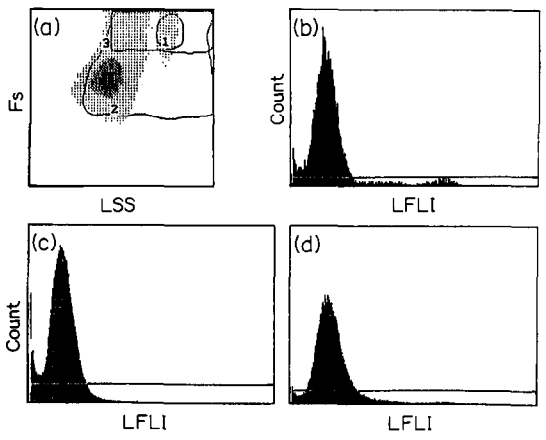

Fig. 1. Reactivity of 1C4 with CBA spleen cells. Cytogram (a) shows three clusters of cells distinguished by forward scattering (FS) and log side scattering (LSS), with three gates: 1 around smallest cluster, 2 around all three and 3 around two smaller clusters. Fluorescence histograms (b), (c) and (d) correspond to gates 1, 2 and 3, respectively.

diameter, reaching 21.8 (S.E. 3.4 mm) on day 22 compared with 5.6 (4.8 mm) in control mice ($P < 0.01$).

These results directly implicate natural cytotoxic cells in surveillance against the WEH1-164 fibrosarcoma *in vivo*, and indicate that the surveillance mechanism does not require previous immunisation. It is important to note, however, that it is possible to immunise mice to the WEH1-164 fibrosarcoma and to demonstrate that this tumour does express tumour-associated antigens which are recognised by T lymphocytes [3]. Furthermore, these data reveal that NC-1.1$^+$ cells make up less than 1% of CBA splenocytes and that these cells mediate all of the natural cytoxicity *in vitro* against WEH1-164.

The lytic mechanism of natural cytotoxicity appears to be dependent on release of tumour necrosis factor [6]. The cell membrane expression of NC-1.1, the effectiveness of 1C4 pretreatment of spleen cells *in vitro* in abolishing natural cytotoxicity and the kinetics of the *in vivo* activity of 1C4 where a single injection reduces NC activity for up to 1 week all argue strongly against NC-1.1 being tumour necrosis factor itself. Therefore the possible mechanisms of action of 1C4 include blocking of the effector/target cell interaction and/or inhibition of production of tumour necrosis factor. We are now trying to distinguish between these two possibilities, to determine the lineage(s) of origin of NC-1.1$^+$ cells and to investigate their other *in vivo* role(s). Stutman *et al.*, who first described NC cells [7], have now shown that natural cytotoxicity against WEH1-164 *in vitro* is mediated by cells of more than one lymphohaemopoietic lineage [8]. We plan further experiments with 1C4 and WEH1-164 to investigate which of these different subpopulations of natural cytotoxic cells mediates tumour surveillance against WEH1-164 *in vivo*.

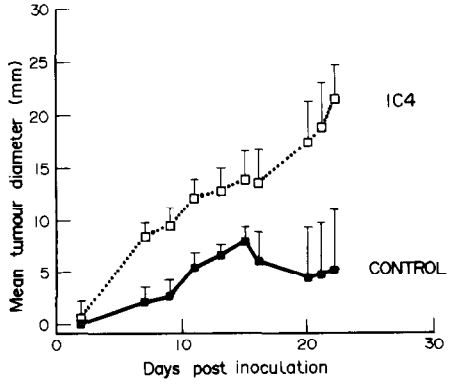

Fig. 2. Effect of 1C4 on growth of WEH1-164 fibrosarcoma (CBA × BALB/c)F_1 mice (mean, S.E.).

1. Burton RC, Smart YC, Tooney PA, Johnston W, Farrelly ML. A monoclonal antibody specific for murine natural cytotoxic cells. *Fed Proc* 1989, **3**, 803.
2. Smart YC, Stevenson KL, Farrelly ML, Brien JM, Burton RC. Production of a monoclonal allo-antibody to murine natural cytotoxic (NC) cells. *Immunol Cell Biol* 1990, **68**, 277–284.
3. Burton RC, Warner NL. *In vitro* induction of tumour specific immunity. V. Detection of common antigenic determinants of murine fibrosarcomas. *Br J Cancer* 1978, **37**, 159–170.
4. Burton RC, Grail D, Warner NL. Natural cytotoxicity of haematopoietic cell populations against murine lymphoid tumours. *Br J Cancer* 1978, **37**, 806–817.
5. Burton RC, Ferguson P, Gray M, Hall J, Hayes M, Smart YC. Effects of age, gender and cigarette smoking on human immunoregulatory T cell subsets: Establishment of normal ranges and comparison with patients with colorectal cancer and multiple sclerosis. *Diag Immunol* 1983, **1**, 216–223.
6. Ortaldo JR, Mason LH, Matheson BJ, Liang SM, Flick DA, Herberman RB. Mediation of mouse natural cytotoxic activity of tumour necrosis factor. *Nature* 1986, **321**, 700–702.
7. Stutman O, Paige CJ, Figarella EF. Natural cytotoxic cells against solid tumours in mice. I. Strain and age distribution and target cell susceptibility. *J Immunol* 1978, **121**, 1819–1826.
8. Bykowsky MJ, Stutman OJ. The cells responsible for murine natural cytotoxic (NC) activity. A multi-lineage system. *J Immunol* 1986, **137**, 1120–1126.

Acknowledgements—This study was supported by the National Health & Medical Research Council of Australia, New South Wales State Cancer Council of Australia, Government Employees Assistance to Medical Research Fund and the Royal Australasian College of Surgeons.