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Specificity of the passive antibody-induced suppression of the humoral immune response of mice to surface antigens on human cells

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Summary. The effect of passively administered antibody on the humoral immune response of BALB/c mice to antigenic determinants on human cells has been examined. Antiserum raised by immunizing mice with the human leukaemic cell line K562, which lacks HLA-A,B,C antigens, was administered to mice, together with the HLA-A,B,C-positive cell line, BALM-1. The antibody response to the unique antigen was assessed by measuring the ability of the resultant antiserum to inhibit the binding to BALM-1 cells of a labelled monoclonal antibody, 7B6, which is specific for a monomorphic HLA-A,B,C determinant. As an indication of the immune response to antigens common to K562 and BALM-1, the ability of the same antiserum to inhibit the binding of monoclonal antibody 6B1, which detects an epitope common to both cell lines, was measured.

Passive antibody to K562 blocked the immune response of mice to the common antigen on BALM-1 cells. However, the response to the antigen not recognized by the passive antibody was unaffected, even though the two antigens were present on the same immunizing cell. Thus, the effect of passive antibody was 'determinant specific'. Similar results were obtained, irrespective of whether the i.v. or i.p. route of immunization was used, and whether the passive antibody was adsorbed onto the immunizing cells prior to injection, or administered separately.

The blocking of the immune response did not depend on simple masking of the antigenic determinants by the passive antibody, since non-saturating amounts of antibody were effective. In addition, blocking activity was dependent on antibody class and on an intact Fc region. The latter considerations also imply that the outcome of passive antibody administration in this system was determined by factors other than the ability of the antigen-antibody complexes to interact directly with B cells, and indicate the importance of antigen processing and/or a mechanism such as antigen-reactive cell opsonization.

INTRODUCTION

Passive administration of antibody, together with an immunogen, is known to block the induction of an immune response (Uhr & Möller, 1968). This principle has been applied in the past in blocking the response to 'normal' cell antigens on tumour cells in order to produce heteroantisera detecting tumour-associated antigens (Weiner, Hubbard & Mardiney, 1972; Greaves *et al.*, 1975; Billing *et al.*, 1980). The hybridoma technology has overcome many of the problems of producing specific antibodies to tumour-associated

Abbreviations: ALS, anti-lymphocyte serum; ARCO, antigen-reactive cell opsonization; BSA, Bovine serum albumin; FCS, fetal calf serum; [^{125}I]UdR, 5-(^{125}I) iodo-2′-deoxyuridine; PBS, Dulbecco's Ca^{2+}-Mg^{2+}-free phosphate-buffered saline, pH 7·4.

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antigens using whole cells as immunogen. Nevertheless, it is necessary to screen many antibodies to find those detecting antigens of interest. The 'passive antibody' approach has the potential to increase the relative yield of hybridomas producing useful monoclonal antibodies.

While passive administration of antibodies to normal cells enabled production of conventional antisera with improved tumour selectivity, there was, at the time, no way of quantitating the relative effect of the passive antibody on the response to masked (i.e. 'normal') and unmasked (i.e. tumour-associated) antigens on the immunizing cells. The question of the determinant specificity of the immunosuppression by passive antibody has been examined in hapten-carrier systems with conflicting results. Using dinitrophenol-substituted human γ-globulin as immunogen, and passive antibody to dinitrophenol or human γ-globulin in guinea-pigs, it was found that the suppression was determinant-specific, provided that the immunizing immune complexes were formed in antigen excess (Henney, 1971). In contrast, Kappler *et al.* (1973) did not observe determinant specificity in a murine system using trinitrophenol-substituted sheep red blood cells as immunogen, together with passive antibody to trinitrophenol or sheep red blood cells.

Results obtained with hapten systems may depend on the level of substitution, and are also complicated by carrier requirements. With the availability of monoclonal antibodies, it is possible to quantitate the humoral immune response to individual natural antigenic determinants on a complex immunogen. Thus, it is possible to study the effects of passive antibody directly in the system of interest, and to determine which immunization protocol gives the best results.

In this study, we have quantitated the antibody responses of mice to individual antigenic determinants on human cells by examining the ability of the polyclonal antisera to compete with labelled monoclonal antibodies for a limiting number of binding sites on the surface of intact cells. Two human cell lines were used: a B-lymphoblastoid line, BALM-1 (Minowada *et al.*, 1977) and a myeloid leukaemia line K562 (Lozzio & Lozzio, 1979). Both cell lines express an antigen detected by our monoclonal antibody 6B1, and share many other antigens as judged by the extent of binding of polyclonal anti-K562 serum to BALM-1. In contrast, HLA-A,B,C antigens are expressed on BALM-1, but not on K562. We have examined the ability of passive antibody raised against K562, and presumably lacking any anti-HLA-A,B,C activity, to block the immune response of mice to shared (6Bl-antigen) and unique (HLA-A,B,C) antigens on BALM-1 cells. The results indicated that blocking of the response to antigens on the cell surface by passive antibody was 'determinant' specific; that is, the response to antigens on the cell which were not recognized by the passively transferred serum was unaffected.

MATERIALS AND METHODS

Mice

Except where otherwise stated, BALB/c mice, bred in the University of Adelaide Medical School Animal House from stock obtained from the Institute of Medical and Veterinary Sciences, Adelaide, were used in these experiments. Mice of both sexes were used at 8–20 weeks of age. In one experiment, specific pathogen-free LACA mice, bred as a closed colony at the University of Adelaide Central Animal House from stock obtained in 1973 from the Medical Research Council, Carshalton, Surrey, U.K. were used.

Cell lines

The sources of cell lines and culture conditions were as previously described (O'Keefe & Ashman, 1982).

Monoclonal antibodies

Hybridomas secreting antibodies 7B6 and 6Bl were prepared and cloned according to standard procedures (Oi & Herzenberg, 1980) using myeloma P3. NS1.Ag4.1 and spleen cells from BALB/c mice which had been immunized with leukaemic blasts from a patient with acute myelomonocytic leukaemia. Based on its pattern of reactivity with a panel of cell lines, 7B6 is believed to bind to a monomorphic HLA-A,B,C determinant, whereas 6B1 binds to all blood cells and cell lines tested. Hybridomas were grown as ascites in pristane-primed BALB/c mice, and the monoclonal antibodies 7B6 and 6B1, respectively IgG2a and IgG3 class, were purified by affinity chromatography on Protein A-Sepharose (Pharmacia, Uppsala, Sweden) as described by Ey, Prowse & Jenkin (1978). The purified antibodies were labelled with ^{125}I-sodium iodide (Amersham International, Amersham, Bucks, U.K.) to a specific activity of approximately 5 μCi/μg by the 'iodogen' method (Salacinski *et al.*, 1981).

Immunization procedures

Cells were harvested from cultures in the logarithmic

phase of growth, washed twice with sterile PBS and resuspended at 5×10^7/ml. Aliquots of 0·2 ml (i.e. 10^7 per mouse) were injected via the i.v. or i.p. route, as indicated. Anti-K562 serum for passive administration was raised by immunizing BALB/c mice with 10^7 K562 cells by the i.p. route three times at 3-week intervals. Mice under ether anaesthesia were bled from the retro-orbital plexus 6 days after the second and third immunizations, and the sera were pooled. ALS was prepared in a similar way using human tonsillar lymphocytes as immunogen. The sera were stored at −20°.

The amounts of anti-K562 and ALS sufficient to saturate the binding sites on BALM-1 cells were determined by indirect radioimmunoassay. BALM-1 cells were harvested, washed as above, and suspended at 10^7 cells per ml in PBS containing 0·1% NaN_3 and 0·1% BSA. Fifty-μl aliquots were incubated with duplicate 50-μl aliquots of serial 1/2 dilutions of anti-K562 serum, ALS or normal mouse serum on ice for 30 min. The cells were washed with 3×1 ml aliquots of PBS-NaN_3-BSA, then incubated with an excess of polyclonal rabbit anti-mouse $(F(ab')_2$ which had been labelled with ^{125}I as described above. After a further 30 min incubation on ice, the cells were washed twice and the cell-associated radioactivity determined using a Packard Auto-Gamma counter. A 'saturating amount' of antiserum was taken as twice the greatest dilution which gave at least 95% of maximum binding, and was 1/10 for the anti-K562 and 1/20 for the ALS.

In some cases, passive antibody was administered by coating the BALM-1 cells *in vitro* prior to injection. Cells were incubated at 10^7/ml in sterile PBS with the appropriate antiserum dilution (100 μl/10^7 cells for anti-K562 or normal mouse serum; 50 μl/10^7 cells for ALS) on ice for 30 min, washed twice with PBS and resuspended at 5×10^7/ml in PBS for injection. In other instances, passive antibody was injected i.v. or i.p. 4–6 hr prior to injection of BALM-1 cells.

Where indicated, the antiserum for passive administration was fractionated into its IgG and IgM components using Protein A-Sepharose. $F(ab')_2$ fragments of the IgG fraction were prepared by digestion with pepsin as previously described (Gadd & Ashman, 1983).

Competitive radioimmunoassay of the polyclonal antisera

The response of mice to the epitopes recognized by monoclonal antibodies 7B6 and 6B1 was measured by determining the ability of the resulting antisera to inhibit the binding of ^{125}I-labelled 7B6 and 6B1 to BALM-1 cells. Sera from five mice per group were pooled and serial 1/2 dilutions in PBS-NaN_3-BSA were tested in duplicate. BALM-1 cells (5×10^5 in 25 μl PBS-NaN_3-BSA) were incubated with 50 μl of appropriately diluted antiserum on ice for 30 min. Labelled 7B6 or 6B1 ($4–7 \times 10^4$ c.p.m. diluted with cold carrier—see below) was then added in 25 μl PBS-NaN_3-BSA and the incubation continued for a further 30 min. The cells were washed with 2×1 ml aliquots of PBS-NaN_3-BSA and the cell-associated radioactivity determined. In order to maximize the sensitivity of the assay, conditions were chosen such that the number of antigenic sites was limiting. This was done by the addition of 'cold carrier' to the labelled antibodies in a quantity sufficient to inhibit the binding by approximately 30%.

Survival of the immunizing cells after injection

The transplantable murine tumour, Ehrlich's Ascites tumour, obtained from Dr I. Kotlarski of this Department, was labelled with [^{125}I]UdR (Amersham) *in vitro* by the method of V. La Posta and I. Kotlarski (personal communication). Briefly, cells from i.p. passage were harvested, washed and adjusted to 5×10^6/ml in medium + 5% FCS. [^{125}I]UdR, 0·038 μCi/10^7 cells, was added, followed by incubation at 37° for 2 hr. Labelled cells were centrifuged through a FCS layer at 400 ***g*** and washed with serum-free medium prior to injection. The human leukaemia cell lines BALM-1, K562 and U937 were radiolabelled by a modification of this procedure. [^{125}I]UdR was added to log phase (5×10^5/ml) cultures of the cell lines at the rate of 0·38 μCi/60 ml culture and incubated under the usual culture conditions for 24 hr. Cells were harvested by centrifugation at 200 ***g***, washed twice and resuspended in PBS for injection. Labelled cells for use as killed controls were heated at 56° for 30 min prior to injection. Lysis of the injected cells *in vivo* was indicated by clearance of the nuclear label, determined by whole-body counting in a NaI crystal scintillation detector (Nuclear Chicago, G.D. Searle and Co., Des Plaines, IL, U.S.A.) as described by Ashley & Kotlarski (1982).

RESULTS

Specificity of the immune suppression by passive antibody

Groups of five mice were immunized with BALM-1 cells (10^7/mouse), with or without saturating levels of

passive mouse anti-K562, ALS or normal mouse serum, according to the following protocols:

(i) BALM-1 cells were treated with antibody prior to injection i.p.;

(ii) as for (i) but injected i.v.;

(iii) passive antiserum (0·25 ml/mouse for anti-K562 and normal mouse serum, 0·125 ml/mouse for ALS) was administered to mice i.p. 4–6 hr prior to injection of BALM-1 cells by the same route;

(iv) as (iii) but using the i.v. route for both antiserum and cells.

Where antisera were administered separately, i.e. (iii) and (iv), the dose used was 2·5-fold higher than that necessary to saturate the cells *in vitro*. In each case, the procedure was repeated after 4 weeks and the mice bled 6 days later. The pooled antisera from each group were assayed for their content of antibodies directed against the epitopes recognized by 7B6 and 6B1 by competitive radioimmunoassay on BALM-1 cells. The results obtained using protocol (iv) are shown in Fig. 1; similar results were obtained with the other three protocols. Sera from mice immunized with BALM-1 cells alone, or with BALM-1 cells plus normal mouse serum, inhibited the binding of both ^{125}I-6B1 (Fig. 1a) and ^{125}I-7B6 (Fig. 1b) to the target cells, and therefore contained antibodies to the epitopes recognized by both monoclonal antibodies. Sera from normal mice, or from mice immunized with BALM-1 cells plus ALS, failed to inhibit the binding of either monoclonal antibody to the cells, and therefore do not contain an appreciable amount of antibody to either epitope. Thus, treatment of mice with passive antiserum to

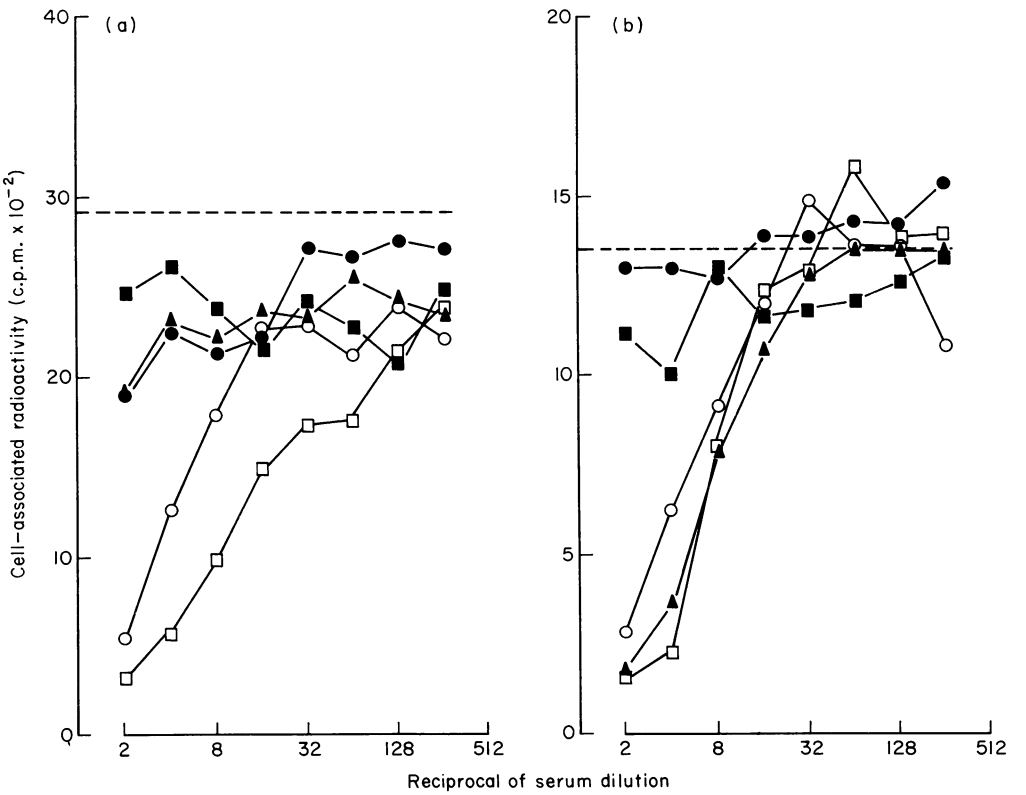

Figure 1. Suppression of the response to masked and unmasked antigens by passive antibody. BALM-1 cells and, where applicable, passive antibody were administered separately by the i.v. route to groups of five mice. The resultant antisera were assayed for specific antibody content by their ability to inhibit the binding of labelled monoclonal antibodies 6B1(a) and 7B6(b) to BALM-1 cells. Normal mouse serum control (■——■); antiserum to BALM-1 cells: alone (□——□), + passive normal mouse serum (○——○), + passive anti-K562 serum (▲——▲), + passive ALS (●——●). The discontinuous line indicates the level of binding in the absence of added serum. Data points are the average of duplicate assays. Throughout the whole series of experiments, the mean difference between duplicates as a percentage of their average was 10·1%.

normal lymphocytes (which express the epitopes detected by both 6B1 and 7B6) blocked the response to those epitopes. In contrast, serum from mice immunized with BALM-1 cells plus passive anti-K562 serum inhibited the binding of ^{125}I-7B6 (Fig. 1b), but not of ^{125}I-6B1 (Fig. 1a) to BALM-1 cells, and therefore contained antibodies to the epitope detected by 7B6, but not to that detected by 6B1. Thus, passive anti-K562 blocked the response to the 6B1 epitope which is shared by K562 and BALM-1, but did not affect the response to the 7B6 epitope which is unique to BALM-1. That is, the suppression was specific at the determinant level, not the particulate level.

Effect of varying the amount of passive antiserum

The experiments described in the preceding section were carried out using relatively large amounts of passively transferred antiserum. Using the i.v. route with administration of antiserum prior to BALM-1 cells, the effect of 10-fold and 100-fold less passively transferred antiserum was examined in groups of five mice. These amounts of antiserum were sufficient to bind approximately 75% and 10%, respectively, of the available antigenic sites on the immunizing dose of BALM-1 cells under the assay conditions described in the Materials and Methods. In each case, the procedure was repeated after 4 weeks and the mice were bled 6 days later. The results shown in Fig. 2 demonstrate that suppression of the response to 6B1 epitope by anti-K562 serum, and of the response to 7B6 epitope by ALS, occurred even at the lowest level of passive antibody tested.

Suppressive ability of different Ig fractions

The IgG fraction of the anti-K562 serum was purified on Protein A-Sepharose, and F(ab')$_2$ fragments were

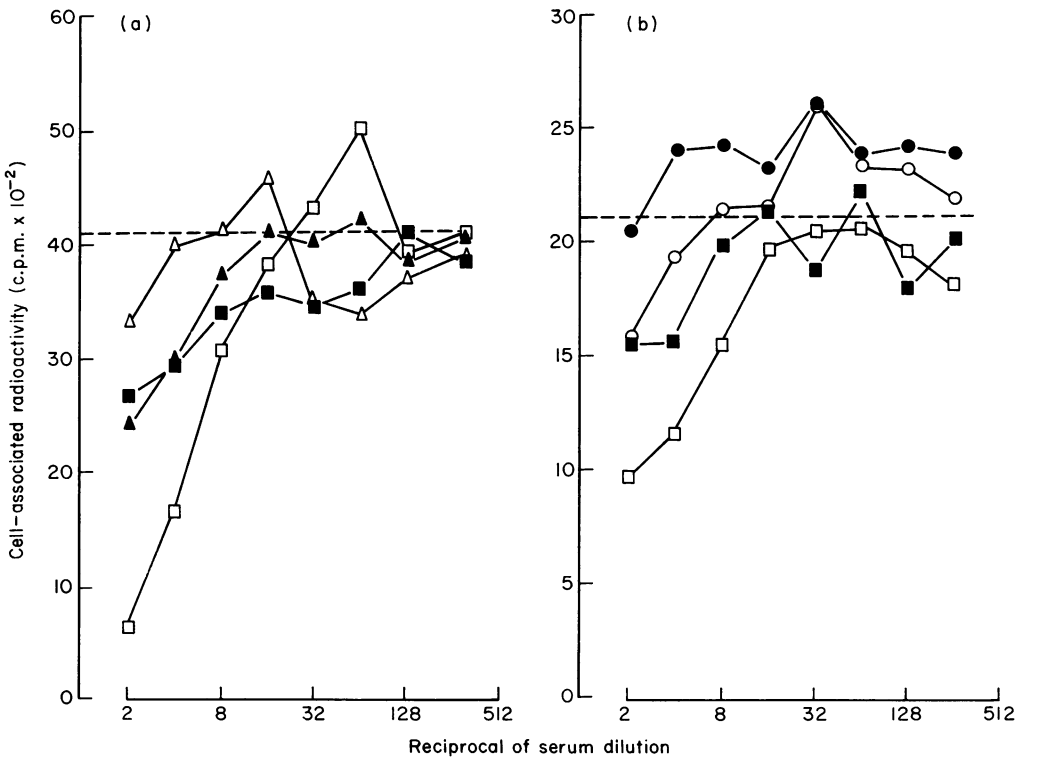

Figure 2. Effect of dose of passive antibody. The experimental conditions were as described for Fig. 1, except that the doses of passive antibody used were 10-fold less (solid symbols) and 100-fold less (open symbols). Inhibition of the binding of ^{125}I-6B1 and ^{125}I-7B6 to BALM-1 cells are shown in (a) and (b), respectively. Normal mouse serum control (■——■); antiserum to BALM-1 cells: alone (□——□), +passive anti-K562 serum (▲,△), + passive ALS (●,○).

prepared from some of it. Whole anti-K562 serum, the IgG fraction, the IgM (i.e. IgG-depleted) fraction, and $F(ab')_2$ fragments of the IgG were administered to groups of five mice by the i.v. route prior to immunization with BALM-1 cells. In each case, the amount of antibody used was proportional to its quantity in the original antiserum. The dose used in this experiment was sufficient to bind approximately 75% of the antigenic sites on the number of BALM-1 cells used for immunization. Secondary immunization was carried out at 4 weeks using the same procedure, and the mice were bled 6 days later. As shown in Fig. 3, the IgM fraction was entirely ineffective in blocking the response to the 6B1 epitope, whereas the IgG fraction blocked as well as the whole serum. $F(ab')_2$ fragments exerted a slight blocking effect, which may have been due to the presence of traces of intact IgG. Thus, the immunosuppression brought about by the passive anti-K562 serum was mediated by the IgG fraction and appeared to be Fc dependent. While the relative amounts of antibody capable of binding to BALM-1 cells in the IgG and IgM fractions was not determined in this instance, it seems unlikely that the lack of immunosuppression by the latter was due to there being insufficient specific antibody in that fraction. As shown in the previous section, the amount of antibody used in this experiment was at least 10-fold higher than that necessary to cause suppression of the response to the 6B1 epitope. In addition, in other work, we have observed a considerable amount of specific IgM antibody production by mice immunized with human cells by the procedure used to prepare the anti-K562 serum.

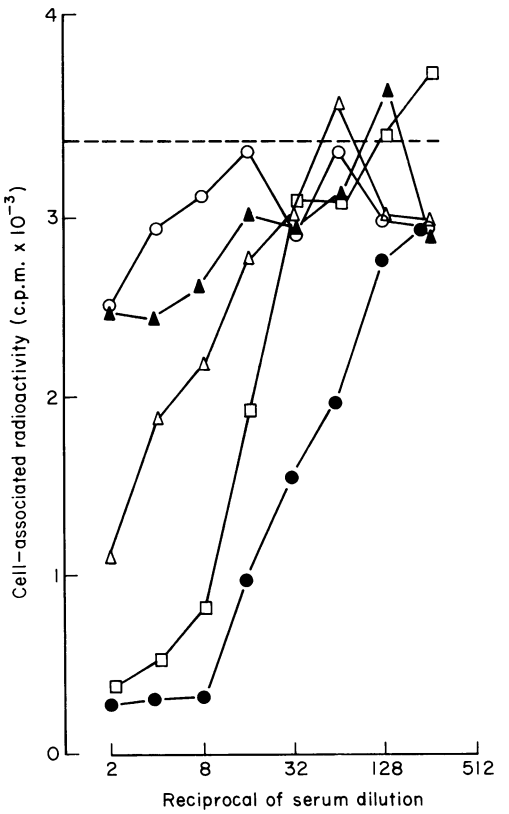

Figure 3. Fraction of the anti-K562 serum responsible for blocking. Experimental conditions were as those described for Figs 1 and 2. The passive antiserum, or fractions prepared from it, were used at the medium dose rate. The figure shows inhibition of binding of ^{125}I-6B1 to BALM-1 cells by antiserum raised by immunizing mice with BALM-1 cells: alone (□——□), + passive anti-K562 serum (▲——▲), + passive anti-K562 IgG (○——○), + passive anti-K562 IgG-$F(ab')_2$ (△——△), + passive anti-K562 IgM (●——●).

Survival of the immunizing cells after injection

At the beginning of this study, we intended to examine the effect of passive antibody on the survival of human cells in the mouse. To this end, BALM-1 cells were labelled with the thymidine analogue [^{125}I]UdR, which is incorporated into the DNA and is released to an appreciable extent only after cell death. Under the experimental conditions used, the released label is not re-utilized, but is rapidly excreted in the urine. Thus, an estimate of the survival of the injected cells in the mouse can be obtained by whole-body radiation counting (Ashley & Kotlarski, 1982).

BALM-1 cells labelled with [^{125}I]UdR to an activity of 500–1500 c.p.m. per 10^6 cells retained greater than 90% viability for at least 24 hr after labelling. All of the cell-associated label was TCA-precipitable, i.e. in high molecular weight form. The results of an experiment in which groups of mice were injected i.p. with 5×10^6 labelled BALM-1 cells, or 10^6 labelled Ehrlich Ascites Tumour cells per mouse, are shown in Fig. 4. Radioactivity was cleared from mice injected with viable BALM-1 cells almost as fast as from mice injected with killed BALM-1 cells. That is, most of the BALM-1 cells survived less than 24 hr (the limit of detection of the assay) after injection. Similar results were obtained usng the i.v. route of injection and with other human leukaemic cell lines, K562 and U937, and when specific pathogen-free LACA mice were used instead of conventional BALB/c mice (data not shown). In contrast, approximately 80% of the radioactivity

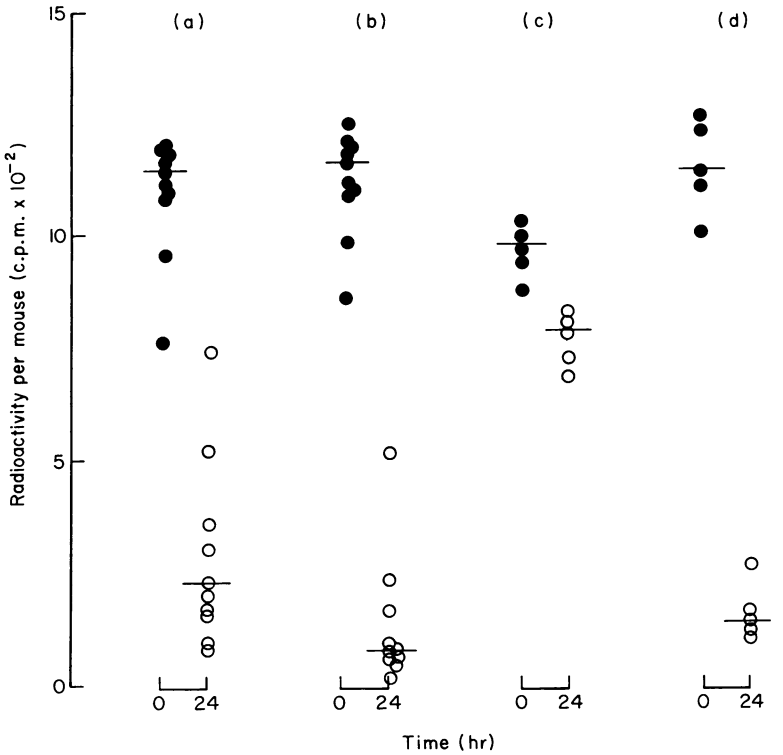

Figure 4. Killing of BALM-1 cells *in vivo*. BALB/c mice were injected i.p. with [^{125}I]UdR-labelled cells as follows: (a) BALM-1 cells, 5×10^6/mouse; (b) heat-killed BALM-1 cells, 5×10^6/mouse; (c) Ehrlich Ascites cells, 1×10^6/mouse; (d) heat-killed Ehrlich Ascites cells, 1×10^6/mouse. Whole-body radiation was measured immediately after injection (solid circles), and 24 hr later (open circles). The figure shows data for individual mice.

associated with viable murine tumour (Ehrlich's ascites) cells was retained 24 hr after injection (Fig. 4).

DISCUSSION

The successful use of passive antibody to normal cell antigens in the preparation of conventional heteroantisera to tumour-associated antigens suggested that the immunosuppression brought about by passive antibody was 'determinant-specific' rather than 'particle (i.e. cell) specific'. We have now provided direct evidence that this is the case. In contrast, enhancement of allograft survival by passive antibody-to-donor cell surface antigens appears to be specific at the 'particulate' level (Hutchinson, 1980). The reason for the difference is not clear: it may be that different mechanisms apply in the two situations, or it may be related to the duration of survival of the foreign cells in the recipient.

Even in the absence of passive antibody, human leukaemic cells injected into mice were killed within 24 hr, which is too rapid to be due to induction of a primary immune response. This result was obtained in two strains of mice, and with three different cell lines, including U937 which is resistant to murine 'natural killer' cells (Haller *et al.*, 1977). Tumouricidal-activated macrophages did not seem to be involved, since murine Ehrlich Ascites tumour cells were not killed. Biochemical incompatibility is not likely to be the explanation, since human tumour cells (including leukaemic cells) can be grown in nude mice. Therefore, it seems likely that the mice had 'natural antibodies' to some human cell antigens. Consistent with this, we frequently observed some inhibition of the binding of ^{125}I-labelled monoclonal antibodies by normal mouse serum. To explain why these 'natural' antibodies did not suppress any subsequent immune response in their own right it is necessary to postulate that they are almost exclusively of IgM class. Whatever the

explanation, rapid lysis of the immunizing cells may have resulted in independent subsequent interaction of the different cell surface molecules with the immune system of the mouse. The passive antibody could, of course, also contribute to rapid cell lysis, although our assay system was not sensitive enough to enable us to test this.

Unlike the hapten-carrier system described by Henney (1971), the determinant specificity observed in our work was independent of the dose of passive antibody over a wide range. As has been observed in the allograft enhancement system (Hutchinson, 1980), the immunosuppressive effect of passive antibody was observed with amounts far less than would have been necessary to saturate the available antigenic determinants, and was dependent on an intact Fc region. A mechanism which has been postulated to explain the enhancement of allograft survival by passive antibody is *antigen reactive cell opsonization* (ARCO), in which macrophages attach to (and kill) the antigen-reactive lymphocytes of the recipient via the antigen-antibody complexes. For cell-associated antigens, this model predicts specificity at the particulate level (Hutchinson, 1980). However, with rapid lysis of the immunizing cells, the ARCO mechanism is also consistent with the determinant-specificity observed in our experiments.

The failure of passive antibodies of the IgM class to block the immune response remains to be explained. In contrast to the binding of IgG-containing immune complexes, binding of IgM-containing immune complexes to mouse macrophages was reported not to result in ingestion, unless the macrophages were activated (Bianco, Griffin & Silverstein, 1975). Thus, it is possible that passive antibody acts by affecting antigen uptake and processing by macrophages.

Antigen-specific immune suppression by immune complexes is also known to operate by direct interaction of the complexes with B cells via the specific antigen receptor (Abbas & Klaus, 1978). However, this mechanism is clearly not the major one operating here, since it has been reported not to depend on the antibody class, nor an intact Fc region.

In conclusion, passive antibody to human leukaemic cells has been shown to suppress the humoral responses of mice in a determinant-specific manner. The two epitopes studied are almost certainly on different molecules on the cell surface. It is not known whether the same results would be obtained with different epitopes on the same molecule. Nevertheless, these experiments provide a sound basis for the future use of passive antibodies in the preparation of conventional or monoclonal xenoantibodies to tumour-associated or differentiation antigens. In addition, the results suggest that the preparation of cell extracts (Al-Rammahy & Levy, 1979) does not offer any advantage, at least where antibodies to human cells are raised in mice.

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