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Attachment of an anti-receptor antibody to non-target cells renders them susceptible to lysis by a clone of cytotoxic T lymphocytes

(T-cell receptor/major histocompatibility complex/H-2 restriction/T-cell-mediated lysis)

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ABSTRACT **The molecular basis for the dependence of antigen recognition by T cells on products of the major histocompatibility complex (MHC) is unknown, and the antigenic structures that are actually bound by T-cell receptors are ill-defined. In this study, we asked whether a monoclonal antibody (mAb) that reacts with the T-cell receptor of a clone of murine cytotoxic T lymphocytes (CTL) and not with the receptors of other CTL clones can substitute for that clone's natural ligand in specific cytolytic reactions. To answer the question, a mAb (1B2) to the receptor of a CTL clone (2C) was attached covalently to ^{51}Cr-labeled cells that were not otherwise susceptible to lysis by clone 2C, and the cells thus modified were then tested as targets for clone 2C and other CTL clones of similar specificity. All labeled cells modified in this way, including a murine cell line that expresses no cell-surface MHC class I molecules and a human cell line, were lysed by clone 2C but not by other CTL clones. If, however, instead of attaching the mAb to the receptor of clone 2C, the cells were modified by attaching to them mAbs to other surface antigens on CTL [lymphocyte function-associated antigen (LFA-1), Thy-1.2], they were not lysed. In cytolytic titrations, the cells that had been converted by attachment of mAb 1B2 into specific targets for clone 2C were just as susceptible to lysis by that clone as the clone's natural H-2^d targets (e.g., P815 cells). However, some accessory surface molecules (LFA-1, Lyt-2) that are required for clone 2C to lyse its natural H-2^d targets seemed not to be required for this clone to lyse the mAb-converted target cells. By demonstrating that a variety of different cell types can be thus converted into target cells for CTL, the approach described in this study may provide opportunities to analyze further the mechanisms by which CTL destroy target cells.**

The specificities of T cells with effector functions (helper and cytotoxic cells) are characteristically dependent on products of genes in the major histocompatibility complex (MHC). Thus, cytotoxic T lymphocytes (CTL) are specific for either (*i*) foreign antigens that are recognized only in the context of self-MHC molecules of class I type or (*ii*) allogeneic MHC class I molecules. One interpretation of the CTL requirement for target cell surface class I molecules is that these molecules provide the site at which CTL deliver the lethal hit that results in lysis of target cells (1). Indeed, the conclusion drawn from one recent analysis of lectin-dependent lysis of target cells by CTL is that even in this putatively antigen-independent process the presence of class I MHC products on target cells is still necessary for their lysis (2).

However, it is not clear if the MHC products are required only for recognition of target cells or also for lysis of these cells. To clarify this issue, we have used a monoclonal antibody (mAb) to the T-cell receptor of a clone of CTL to ask whether this mAb can substitute, on target cells, for the CTL's natural ligand in the cytolytic reaction, and, if so, whether the target cell's MHC class I molecules are indispensable for target cell lysis.

The experimental design used to answer these questions consisted of the following steps. First, a mAb was produced against a CTL clone (clone 2C). This mAb was shown to block clone 2C's cytolytic activity and to immunoprecipitate its T-cell receptor, but not to block the activity or to precipitate the corresponding receptors of other CTL clones of similar specificity. Next, the anti-receptor mAb was purified and attached covalently to various ^{51}Cr-labeled cells, which were then tested as target cells in the standard cytolytic assay. The cells to which the mAb was attached (called converted targets to distinguish them from natural target cells) included a human cell line and a murine cell line that lacks cell surface MHC class I molecules. All of the cells converted by mAb 1B2 were lysed by clone 2C and not by the other CTL clones. These results show that target cell surface class I MHC molecules are not necessary for specific cytolysis. When cells were modified by attaching to them mAbs specific for other CTL surface antigens [lymphocyte function-associated antigen (LFA-1), Thy-1.2], they did not become susceptible to lysis; hence, perturbation of the CTL's T-cell receptor seems to be necessary for triggering the cytolytic pathway.

MATERIALS AND METHODS

Mice. BALB/c AnN (H-2^d) and BALB.B (H-2^b) mice were produced in the Center for Cancer Research, Massachusetts Institute of Technology.

Tumor Cell Lines. P815 (H-2^d), EL4 (H-2^b), BW5147 (H-2^k), R1.1 (H-2^k), R1.E (a variant of R1.1 that is negative for MHC class I surface antigens; see ref. 3), HPB-ALL (a human T-cell lymphoma; see ref. 4), and X63.653 (a BALB/c-derived myeloma) were all maintained in culture with RPMI 1640 medium containing 10% fetal calf serum/10 mM Hepes/2 mM L-glutamine/100 units of penicillin per ml/100 μg of streptomycin per ml/50 μM 2-mercaptoethanol.

Cloned CTL. Alloreactive CTL clones 2C, G4, and 2.1.1 were produced from spleen cells of BALB.B mice as described (5). The clones were maintained by weekly stimulation with irradiated (4000 rads) P815 cells plus supernatants from rat spleen cells that were maintained in culture for 48 hr in the presence of concanavalin A (Vector Laboratories, Burlingame, CA).

Monoclonal Antibodies. The mAb (1B2) that recognizes the T-cell receptor of clone 2C was produced as described (6). BALB/c AnN mice were injected intraperitoneally 6 times at 2- to 3-week intervals, each time with 10–20 $\times$ 10^6 cells (clone 2C). Four days after the final injection, spleen cells were harvested and fused with X63.653 myeloma cells, using 50% polyethylene glycol. Cells were distributed into ten 24-

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Abbreviations: CTL, cytotoxic T lymphocytes; LFA, lymphocyte function-associated antigen; mAb, monoclonal antibody; MHC, major histocompatibility complex.

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well plates (Costar), and grown in hypoxanthine/aminopterin/thymidine selection medium. After 2–3 weeks, culture supernatants were screened for their ability to block lysis of P815 target cells by clone 2C in a ^{51}Cr release assay (see below).

Rat mAbs against lymphocyte function-associated antigen type 1 (LFA-1; 4-16-1) and class I-H-2^b surface antigens (3-18-8) were produced as described (6). Rat mAb against Lyt-2 (3.155.2) was a generous gift from Frank Fitch and Marian Sarmiento (University of Chicago) (7).

Cytotoxicity Assays. CTL-mediated target cell lysis was measured by a standard ^{51}Cr release assay (8). Various numbers of CTL (in 100 μl of medium) were added to 2×10^4 ^{51}Cr-labeled target cells (in 100 μl) and incubated at 37°C for 4 hr. Cells were pelleted by centrifugation, supernatants were assayed for radioactivity, and percentage specific ^{51}Cr release was calculated from $100 \times (a - b)/(t - b)$, where a is ^{51}Cr release in the presence of CTL, b is the spontaneous release from labeled target cells in the absence of the CTL (<15%), and t is the total ^{51}Cr content of the target cells. In some experiments, CTL were preincubated with mAbs for 30 min prior to addition of ^{51}Cr-labeled target cells. All assays were performed in triplicate.

Purification and Coupling of mAb 1B2. Antibodies were purified from ascites fluid that had been elicited by intraperitoneal injection of BALB/c mice with 5×10^5 viable 1B2 hybridoma cells. Lipoproteins were removed by adsorption to sodium dextran sulfate (0.25%) and precipitation with $CaCl_2$ (1.5%). The enriched γ-globulin fraction, obtained by precipitation in 50% saturated ammonium sulfate, was dialyzed against 50 mM potassium phosphate (pH 8.0). Since mAb 1B2 contains γ1 heavy chains (determined by indirect radioimmune binding assays), the antibody was further purified by anion exchange chromatography on DEAE-cellulose. The pass-through from the DEAE-cellulose contained the antibody and was dialyzed against phosphate-buffered saline (pH 7.4) (P_i/NaCl).

Various procedures for attaching mAbs to target cells are still being examined and characterized. One effective method used in this study consisted of cross-linking the mAb to the cell surface with glutaraldehyde at 0.1%. In this procedure, ^{51}Cr-labeled cells were washed 2 times with RPMI 1640 medium, resuspended in 0.25 ml of RPMI 1640 medium containing 0.2% glutaraldehyde (Baker), and 0.25 ml of mAb 1B2 (2 mg/ml) in P_i/NaCl was added. After a 20-min incubation on ice, the cells were washed with P_i/NaCl and layered over a Ficoll gradient. After centrifugation (3000 rpm; 10 min), the interphase (viable) cells were collected, washed 3 times with RPMI 1640 medium containing 10% fetal calf serum and used in the cytotoxicity assay.

For use in immunoprecipitation assays, mAb 1B2 was coupled to Affi-Gel 10 (Bio-Rad) at ≈5 mg per ml of packed gel.

Labeling of Cell Surface Proteins with ^{125}I. As described previously (6), cloned CTL ($2–3 \times 10^7$) were washed 3 times with P_i/NaCl, and the following were added to the cell pellet: 100 μg of lactoperoxidase, 1 mCi of $Na^{125}I$ (1 Ci = 37 GBq; New England Nuclear), and 50 μl of H_2O_2 (0.03%). Labeled cells were washed 3 times with P_i/NaCl and extracted with 1 ml of 0.5% Nonidet P-40 in 0.15 M NaCl/0.02% NaN_3/25 μM phenylmethylsulfonyl fluoride/10 mM Tris·HCl, pH 7.2 (extraction buffer).

Immunoprecipitation and NaDodSO$_4$/PAGE. ^{125}I-labeled cell extracts (≈1×10^6 cpm), containing 0.5% bovine serum albumin, were incubated with 50 μl of packed 1B2-Affi-Gel 10 for 3 hr on ice. The gel was then washed 3 times with extraction buffer and heated at 100°C in NaDodSO$_4$/PAGE sample buffer, with or without addition of 2-mercaptoethanol (0.1 M). Electrophoresis was performed in 10% polyacrylamide gels containing NaDodSO$_4$ (9). Gels were fixed, dried, and exposed to Kodak X-Omat XAR film using a Dupont Cronex Lightning Plus intensifying screen.

RESULTS AND DISCUSSION

The CTL clones used in this study (2C, G4, and 2.1.1) were derived from BALB.B (H-2^b) spleen cells. Two of the clones (2C and G4) were previously shown in cytolytic assays to be specific for the D end of the H-2^d haplotype (6). The third clone (2.1.1) has a similar specificity (data not shown). To produce a mAb to the T-cell receptor, cells of clone 2C were repeatedly injected into BALB/c mice as described (6), and spleen cells from mice with relatively high titer clone-specific antisera (inferred from inhibitory activity in cytolytic assays) were used to make the BALB/c hybridoma that produces mAb 1B2. Hybridoma culture supernatants containing this mAb inhibited 80% of clone 2C-mediated lysis of P815 target cells (tested at a CTL/target cell ratio of 1:1), whereas under the same conditions these supernatants had no detectable inhibitory activity of the two other clones, G4 and 2.1.1.

The clone-specific activity of mAb 1B2 suggested that it recognized the T-cell receptor of clone 2C (and probably the combining site of this receptor). That this is indeed the case was demonstrated by the ability of this mAb to immunoprecipitate from ^{125}I-labeled 2C cells, but not from similarly labeled clone G4 and 2.1.1 cells, a cell surface molecule with the characteristics of the putative T-cell receptor (10–12): i.e., in NaDodSO$_4$/PAGE the precipitated component had an apparent M_r of 80,000–90,000 under nonreducing conditions and a M_r of 40,000–45,000 under reducing conditions (Fig. 1).

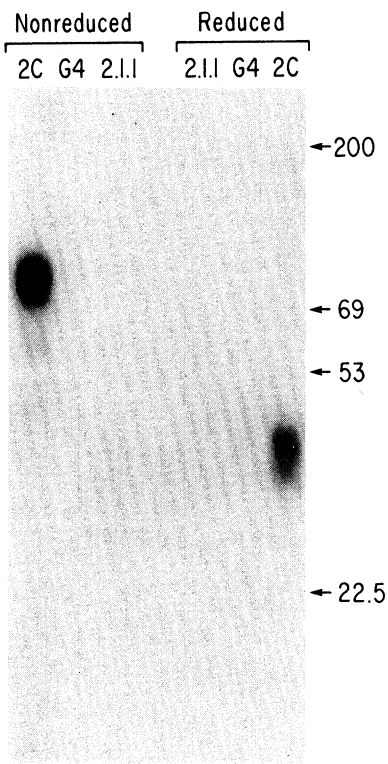

FIG. 1. Immunoprecipitation of the putative T-cell receptor by mAb 1B2 to CTL clone 2C. CTL clones 2C, G4, and 2.1.1 were surface-labeled with ^{125}I, extracted with 0.5% Nonidet P-40, and the labeled extracts were incubated with mAb 1B2 Affi-Gel beads. Material that bound to the beads was removed at 100°C in NaDodSO$_4$/PAGE sample buffer with (reduced) or without (nonreduced) 0.1 M 2-mercaptoethanol and was electrophoresed through a 10% polyacrylamide gel containing NaDodSO$_4$. Molecular weight markers ($\times 10^{-3}$) are myosin (200), bovine serum albumin (69), and immunoglobulin heavy (53) and light (22.5) chains.

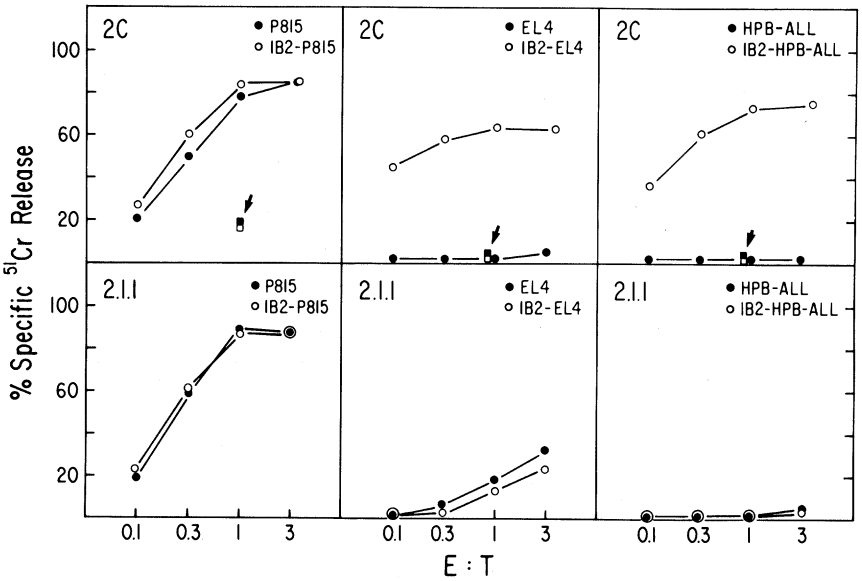

FIG. 2. Cells converted into targets by mAb 1B2 are lysed by CTL clone 2C but not by clone 2.1.1. P815 (an H-2^d murine plasmacytoma), EL4 (an H-2^b murine T-cell lymphoma), and HPB-ALL (a human T-cell lymphoma) were ^{51}Cr-labeled and incubated for 20 min with 0.1% glutaraldehyde/anti-2C mAb 1B2 (1 mg/ml) (○) or with 0.1% glutaraldehyde alone (●): the former (○) are called modified and the latter (●) are called unmodified (see also Fig. 3). After these ^{51}Cr-labeled cells were washed, they were incubated with clone 2C (*Upper*) or clone 2.1.1 (*Lower*) at various effector/target cell ratios (E:T). Arrows indicate experiments in which clone 2C was preincubated with 10 μg of soluble mAb 1B2 per ml before addition of unmodified (■) or 1B2-modified target cells (□).

To test the effect of attaching mAb 1B2 to target cells, two murine cell lines, P815 (H-2^d) and EL-4 (H-2^b), and a human cell line (HPB-ALL) were ^{51}Cr-labeled and then modified by coupling the mAb to them, using glutaraldehyde as the cross-linking reagent. Modified and unmodified cells of each type were then tested for susceptibility to lysis by CTL clones 2C and 2.1.1. As shown in Fig. 2, both clones lysed the modified and unmodified P815 cells, their natural (H-2^d) targets, to an equal extent. Unmodified cells that lack the H-2^d haplotype (e.g., murine EL4 and human HPB-ALL cells) were lysed poorly, if at all, by clones 2C or 2.1.1. However, after attaching mAb 1B2 to these cells, they were lysed extensively by the clone for whose receptor this mAb is specific (clone 2C), but not by the other clone (2.1.1). It is important to note that attachment of mAbs specific for other CTL surface molecules were incapable of similarly converting cells that are not normally susceptible to lysis into cells that are susceptible. For instance, when a mAb to LFA-1 (4-16-1; see ref. 6) and a mAb to Thy-1.2 (13-4; see ref. 13) were attached to EL-4 and to BW5147 (H-2^k) cells, these cells did not become susceptible to lysis by clone 2C (data not shown).

Thus, cells that are not normally susceptible to lysis by a

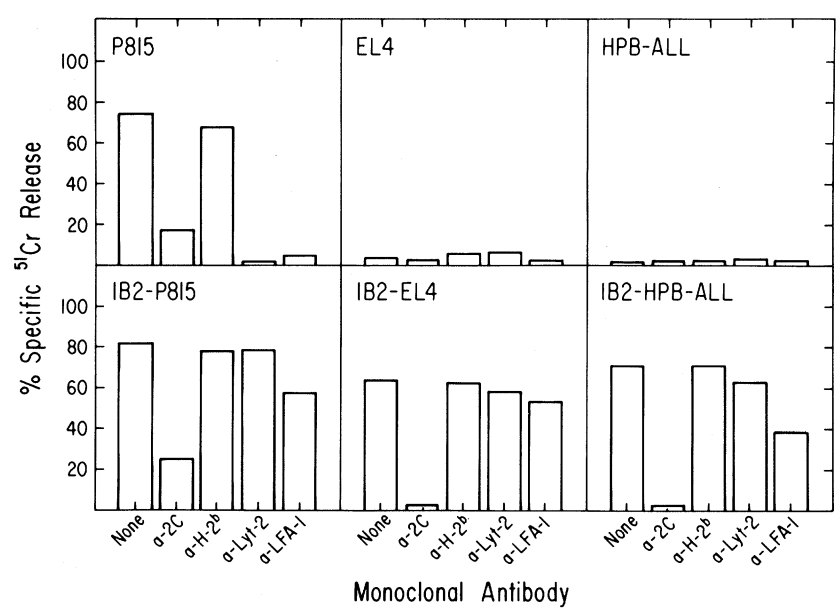

FIG. 3. Effect of various mAbs on the cytolysis of mAb 1B2-modified and unmodified target cells by CTL clone 2C. CTL clone 2C (2×10^4 cells per 50 μl per well) was incubated with 50 μl of hybridoma supernatants containing mAb against either (*i*) the putative antigen-specific receptor of clone 2C (1B2), (*ii*) H-2^b (3-18-8), (*iii*) Lyt-2 (3.155.2), or (*iv*) LFA-1 (4-16-1). After 30 min, 1B2-modified (1B2-P815, 1B2-EL4, 1B2-HPB-ALL) or unmodified (P815, EL4, HPB-ALL) ^{51}Cr-labeled target cells were added (2×10^4 cells per 100 μl per well). After a 4-hr incubation at 37°C, cells were pelleted by centrifiguation and 75 μl of supernatant was assayed for radioactivity. Percent specific ^{51}Cr release was calculated as described.

particular CTL clone become susceptible when they have attached to their surface a mAb to the T-cell receptor of that clone. In contrast, the attachment of mAbs to two other surface molecules of the CTL did not convert nontarget cells into target cells. It is likely, therefore, that the conversion of nontarget into target cells for CTL requires not only that the mAb used to modify target cells bring about bridging between CTL and potential target cells but that is also causes some kind of perturbation (perhaps cross-linking) of the CTLs antigen-binding receptor. The unidirectional killing observed some years ago on mixing two heterogeneous CTL populations in which one recognizes the other, but not vice versa, also suggested that for a CTL to be cytolytically active its antigen-specific receptor must be bound to a ligand (14). Previous studies have also shown that anti-receptor mAbs in the presence of interleukin-2 can cause T-cell clones to proliferate and/or secrete immune interferon (IFN-γ; see refs. 15–17). Thus, similar initial triggering mechanisms may be involved in activating T cells for various processes (target cell lysis, proliferation, IFN-γ secretion).

Specific lysis of target cells by CTL normally requires participation of various antigen-nonspecific accessory molecules on the CTL surface. The two best studied molecules of this type are LFA-1 (18) and Lyt-2 (19, 20). Antibodies to LFA-1 block target-cell lysis by all CTL clones and antibodies to Lyt-2 block lysis by many, but not all, of these clones. In accord with this general experience, mAbs to both LFA-1 and to Lyt-2 (but not a mAb to H-2^b) almost completely blocked clone 2C's lysis of P815 cells, the clone's natural H-2^d target (Fig. 3). However, when converted cells were used as targets (i.e., 1B2-modified P815, EL-4, and HPB-ALL cells) the blocking effect of anti-Lyt-2 was eliminated and that of anti-LFA-1 was reduced (Fig. 3). It is likely that the function of LFA-1 and Lyt-2 is to stabilize CTL–target cell conjugates by reinforcing specific interactions between receptor molecules on CTL and ligands on target cells. The necessity for cooperative effects of LFA-1 may be reduced and that of Lyt-2 may be eliminated, if the affinity of the mAb for the T-cell receptor of clone 2C were much greater than the affinity of that receptor for its natural class I ligand (D^d or L^d). The idea that LFA-1 and Lyt-2 are involved in strengthening the adhesion between CTL and target cells has been suggested (21). All of these findings, illustrated by the data summarized in Fig. 3, emphasize the need to develop methods to measure affinities of T-cell receptors for their ligands on target cells.

Since a human cell line could be effectively converted by the monoclonal anti-T-cell receptor antibody into a target for a clone of murine CTL, it seemed likely that any cell might be similarly converted, even those without detectable cell-surface MHC class I molecules. To explore the latter possibility, we tested R1.1 and R1.E cell lines. R1.1 is a cultured C58/J lymphoma (H-2^k) whose variant R1.E has been shown not to produce β_2-microglobulin and to be devoid of cell surface class I molecules (3). As shown in Table 1, after attachment of mAb 1B2, both R1.1 cells and R1.E cells became equally susceptible to lysis by clone 2C. However, neither 1B2-modified nor unmodified R1.1 or R1.E cells was lysed by the control clone 2.1.1. It appears, therefore, that MHC class I molecules on target cells are not essential for the cytolytic reaction, even when this reaction depends on the CTL's antigen-specific receptor. It is very likely that specific binding to the CTL receptor is the only function for MHC class I molecules in the natural cytolytic reaction.

The ability of anti-receptor antibodies to render a wide variety of cells susceptible to attack by CTL may provide useful opportunities to analyze the mechanisms by which these T cells destroy their target cells. The findings described here also suggest several strategies for eliminating, through the use of CTL, a variety of undesirable cells *in vitro* and *in vivo*.

Table 1. Cytotoxic T cells lyse mAb 1B2-converted H-2^+ and H-2^- target cells*

Cytotoxic T cells			% specific ^{51}Cr release from target cells			
Clone	E/T†	mAb‡	R1.1	1B2-R1.1	R1.E	1B2-R1.E
2C	1:1	—	0.8	50.3	−1.2	45.2
	1:1	1B2	0.7	1.7	−0.2	0.3
	5:1	—	0.1	58.9	−0.3	59.8
	5:1	1B2	0.4	1.3	−2.0	−1.0
2.1.1	1:1	—	0.2	1.3	−0.6	0.8
	1:1	1B2	0.0	1.1	−0.7	−0.3
	5:1	—	0.1	1.3	−1.6	0.4
	5:1	1B2	0.0	1.9	−0.6	−0.5

*R1.1 cells have surface H-2 (H-2^k); the R1.E variant lacks surface H-2 (3).
†Ratio of CTL [effector cells (E) to target cells (T)].
‡Where indicated, soluble mAb 1B2 was added to the cytolytic reaction mixture.

Note Added in Proof. During the preparation of this manuscript, Lancki and Fitch (22) reported that a hybridoma cell line (384.5.32) that secretes a clonotypic anti-T-cell receptor mAb could be lysed by the CTL clone for which the clonotypic mAb was specific, even though the hybridoma does not express the target cell antigen (L^d) recognized by the CTL. Their study, like ours, also showed that mAb to Lyt-2 was effective in blocking lysis of the CTL's natural target cells (L^d) but not lysis of the hybridoma cells (384.5.32).

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