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A HUMAN CYTOTOXIC T CELL LINE WITH RESTRICTED SPECIFICITY FOR SQUAMOUS CELL CARCINOMA

（ヒト扁平上皮癌に対する細胞障害性Ｔ細胞の解析）

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A HUMAN CYTOTOXIC T CELL LINE WITH RESTRICTED SPECIFICITY FOR SQUAMOUS CELL CARCINOMA

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ABSTRACT

Human cytotoxic T lymphocyte (CTL) lines with specificity restricted for autologous squamous cell carcinoma of the head and neck (SCCHN) were established from peripheral blood lymphocytes (PBL) obtained at the time of surgery and again at two different times after surgery from a patient with cancer of the tongue. The CTL lines were cultured in the presence of interleukin 2 (IL2), interleukin 4 (IL4) and autologous tumor (AuTu) cell monolayers. All three lines were $CD3^+CD8^+CD11b^-HLA\text{-}DR^+$ T cell receptor (TCR)α/β^+. They were tested in 4h ^{51}Cr-release assays against SCCHN cell lines (n=5) and a variety of non-squamous human tumor (n=5) and normal (n=5) cell targets and was found to lyse only AuTu (PCI-50) and three allogeneic SCCHN cell lines. Lysis of AuTu and the three allogeneic SCCHN targets by the established CTL lines appeared to be MHC class I restricted, since it was blocked by monoclonal antibodies to class I MHC antigens (Ags). The CTL lines proliferated in vitro in response to autologous PCI-50 or an allogeneic SCCHN cell line (PCI-1). The lines have been maintained in culture in the presence of AuTu monolayers and retained cytotoxicity against AuTu for over 20 weeks.

The AuTu (PCI-50) cell line was tested for in vitro sensitivity to cytotoxic or cytostatic effects of various effector cells, including the CTL lines. PCI 50 targets were resistant to lysis by resting human mononuclear cells but sensitive to IL2-activated natural killer (A-NK) cells in 4h ^{51}Cr-release assays. In comparison with A-NK cells, the CTL line mediated lower levels of lysis against AuTu. Growth of PCI-50 cells in culture was significantly inhibited by a combination of interferon gamma (IFNγ) and IL2 or by high concentrations of tumor necrosis factor alpha (TNFα). While supernatants of A-NK cells were growth inhibitory, those of the CTL line were not. On the other hand, lysis of AuTu targets by the CTL line was increased by preincubation of the tumor cells with TNFα or IFNγ. These cytokines augmented expression of HLA-class I, HLA-class II and intercellular adhesion molecule I (ICAM-I), but not SCC-associated Ags, E7 and A9, on PCI-50 cells. The CTL lines described are the first with restricted specificity for autologous SCCHN ever reported and their availability will facilitate studies of the AuTu T-cell response in head and neck cancer.

INTRODUCTION

Squamous cell carcinoma of the head and neck (SCCHN) cell lines or fresh tumor cells are generally resistant to lysis by non-activated immune effector cells such as peripheral blood natural killer (NK) cells or T lymphocytes obtained from normal volunteers (1). Also, fresh peripheral blood lymphocytes (PBL), lymph node lymphocytes (LNL) or tumor-infiltrating lymphocytes (TIL) obtained from patients with SCCHN have not able to lyse SCCHN targets in 4h ^{51}Cr-release assays (2-4). However, these tumor cells have been shown to be quite sensitive in vitro to lymphokine-activated killer (LAK) cells, IL2-activated human effector cells, conditioned media of A-NK cells (5) or cytokines such as IL2, IFNγ or TNFα (6,7). In a nude mouse model of human SCCHN, growth of the tumor could be effectively inhibited by cytokine-activated human effector cells or their soluble products (5,8). Furthermore, we have recently demonstrated that SCCHN are able to activate fresh or cultured human NK cells (5). As a result of such activation in vitro, NK cells were shown to up-regulate expression of activation Ags, levels of mRNA for various cytokines, antitumor cytotoxicity and proliferation. These results suggest that non-MHC-restricted immune responses elicited by human SCCHN can play a major role in tumor growth inhibition both in vitro and in vivo, in a nude mouse xenograft model.

The role of CTL in antitumor immune response to SCCHN is not known. Although these tumors are generally well infiltrated by T lymphocytes, it has not yet been possible to demonstrate the presence of AuTu-specific CTL among the TIL, LNL or PBL-T cells in patients with SCCHN. This is in contrast to patients with other types of cancer, where the presence of CTL specific for AuTu in peripheral blood or tumor has been recently demonstrated in a variety of human carcinomas, including metastatic melanoma (9,10), ovarian cancer (11,12), renal cell carcinoma (13), gastric cancer (14), glioblastoma (15) or pancreatic cancer (16). These antitumor CTL have been mainly $CD3^+CD8^+TCR\alpha/\beta^+$ and MHC-class I restricted. In melanoma, and perhaps in renal cell carcinoma, antitumor-reactive CTL appear to be important

for successful adoptive immunotherapy (AIT) with TIL (17,18). In vitro ability to lyse AuTu has been shown to correlate with clinical responses to TIL therapy in patients with metastatic melanoma (20). From previous studies, it is unknown whether SCCHN are sufficiently immunogenic to induce a CTL response in vivo or whether immune T cells play any role in resistance to the growth of such tumors. To the best of our knowledge, it has not been so far possible to generate effector cells specific for AuTu from lymphoid cells of patients with head and neck cancer.

In this manuscript, we report the generation and characterization of CTL with specificity restricted to AuTu and a small number of allogeneic SCCHN. Such CTL could be repeatedly generated from the peripheral blood of a patient with SCC of the tongue, indicating that the CTL precursors were present in the patient's peripheral blood for at least 2 years after surgery. Our data indicate that specific T cell responses to AuTu can be detected in at least some patients with SCCHN.

MATERIALS AND METHODS

Patient. The patient was a 92-year-old male with SCC of the tongue (T2N0M0), who underwent partial glossectomy at the University of Pittsburgh Medical Center, Eye and Ear Hospital (Pittsburgh, PA). Histologically the tumor was a well differentiated SCC.

PBL isolation. PBL of the patient were isolated by Ficoll-Hypaque centrifugation prior to surgery and then at two other times, at 18 months and 20 months, after surgery. PBL were examined for viability, counted and cryopreserved.

Establishment of autologous SCC cell line (PCI-50). The fresh tumor specimen obtained under sterile conditions was washed 3 times in Hanks' balanced salt solution (Gibco, Grand Island, NY) containing streptomycin (100 μg/ml), penicillin (100 IU/ml), and amphotericin B (1 μg/ml). Washed tumor tissue was trimmed of fat and necrotic material and finely minced with scalpels into 1- to 2 mm^3 fragments in a sterile Petri dish (Costar, Cambridge, MA). The

fragments of tissue in tissue culture medium (TCM) were transferred to T25 tissue flasks and were maintained undisturbed for 1 week at 37°C in a humidified atmosphere of 5% CO_2 in air. The TCM used was Eagle's minimal essential medium (MEM) supplemented with 1% (v/v) nonessential amino acid mixture, 2 mM glutamine, 100 μg/ml of streptomycin, 100 IU/ml of penicillin, and 15% (v/v) fetal calf serum (FCS) (Gibco) prescreened for *Mycoplasma* and viruses. Flasks containing tumor tissue fragments were observed weekly for evidence of growth in an inverted-phase microscope. Fibroblasts outgrowing from explants were removed at weekly or biweekly intervals, using a cell scraper (Costar No. 3010) and "differential trypsinization" with 0.05% (w/v) trypsin (Gibco) in 0.02% (w/v) EDTA, as described earlier (1). Cultures of cells with the epithelial morphology were extensively washed with TCM following each trypsinization and incubated further in the presence of TCM. The cultures were incubated until epithelial cell monolayers became confluent. They were trypsinized at confluence and split at the 1:2 or 1:3 cell suspension:TCM ratio. Once established, the PCI-50 cell line was maintained in MEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, and antibiotics (see above).

Growth of PCI-50 in nude mice. Athymic BALB/c female 6 week-old mice were obtained from Taconic Farm, Germantown, NY and maintained under pathogen-free conditions in the animal facility. The mice were splenectomized 2 weeks before tumor injections. One day prior to inoculation of tumor cells, all mice were injected intraperitoneally with cyclophosphamide (200 mg/kg; 4 mg/0.1 ml/mouse, Sigma, St. Louis, MO) and anti-asialo GM1 antibody (10 mg/kg; 0.2 mg/0.2 ml/mouse, Wako, Dallas, TX). To establish the tumor, $5x10^6$ PCI-50 cells were injected subcutaneously (sc) in the right flank of each mice in a group of five animals. The tumors were harvested 6 weeks after injection of PCI-50 cells and submitted for histologically examined.

Generation of CTL against PCI-50. The patient's cryopreserved or fresh PBL were used as a source of CTL precursors. The cells were counted and checked for viability using trypan

blue dye. The lymphocytes were cultured in serum-free AIM-V (Gibco) medium in the presence of IL2 (300 IU/ml, Cetus Corp., Emeryville, CA), IL4 (300 U/ml, Immunex Corp., Seattle, WA), and PHA-P (5 μg/ml, Sigma) for 2 weeks in 24-well plates (Costar). After 2 weeks of primary culture, PBL were co-cultured either with autologous irradiated (8,000 R), which were added to cultures once a week, or with viable PCI-50 cells at the lymphocyte (L):tumor (T) ratio of 5:1 in AIM-V medium supplemented with 300 IU/ml of IL2 and 300 U/ml of IL4. Fresh medium supplements were added twice a week. In cultures containing viable tumor cells, PBL appeared to grow well and when their number was greater than $3x10^6$/ml, lymphocytes were transferred to a T-25 flask (Costar) containing partially confluent (30 to 50%) viable tumor cell monolayers. The L:T ratio was maintained at 5:1. Cultures were monitored for the cell number, phenotype and cytotoxicity at regular intervals.

Generation of IL2-activated NK (A-NK) cells. A-NK cells were prepared from allogeneic PBL obtained from normal donors as previously described by us (19). Briefly, monocyte-depleted PBL at a concentration of $5x10^6$ cell/ml were incubated in TCM containing 6,000 IU/ml of rIL2 (Cetus) in plastic flasks for 24h. At the end of the incubation period, the plastic-adherent cells were supplemented with TCM containing 6,000 IU/ml IL2 and co-cultured in the presence of mitogen-prestimulated and irradiated PBL to generate a population highly enriched in activated NK (A-NK) cells. These cells were maintained in culture at a concentration of $1.5\text{-}2x10^6$ cells/ml by supplying fresh TCM containing 6,000 IU/ml IL2 as needed. The cell cultures and supernatants were harvested between days 10 and 14 of growth.

Target cells. In addition to the autologous SCCHN cell line (PCI-50), 4 allogeneic SCCHN cell lines: PCI-1, -2, -4A, -4B (1), as well as K562, a chronic myelogenous leukemia cell line; Daudi, B cell lymphoma; LP and SW, cholangiocarcinomas (20); HR, gastric cancer (14); normal fibroblasts (n=2), and normal keratinocytes (n=3) were used as target cells for cytotoxicity assays. The cell lines were maintained in culture as described earlier (1) and passaged by trypsinization.

Cytotoxicity assays. Cytotoxicity of PBL and CTL was determined using 4h miniaturized ^{51}Cr-release assays as described earlier (2). Briefly, $1x10^3$ cell targets labeled with ^{51}Cr (5 μCi/ml; New England Nuclear, Boston, MA) were plated in triplicate in wells of a 96-well V-bottom plate (Costar) and mixed with effector cells at E:T ratios ranging from 25:1 to 3:1. Cells were centrifuged at 1000 rpm for 5 min and incubated for 4h at 37°C in a CO_2 incubator. The amount of ^{51}Cr released into the supernatant (20 ml) was measured using a beta counter (LKB, Pharmacia, Gaithersburg, MD). Maximal radioisotope release was determined in wells containing target cells only after addition of 5% (v/v) Triton X-100. The percentage of specific lysis was determined as:

$$\% \text{ specific lysis} = \frac{\text{mean experimental cpm - mean spontaneous cpm}}{\text{mean maximal cpm - mean spontaneous cpm}} \times 100$$

Lytic units (LU) of cytotoxicity were calculated according to the method of Pross et al (21). One LU was defined as the number of effector cells required for 20% lysis of $1x10^3$ target cells, and the number of lytic units present in 10^7 effector cells was calculated.

In blocking experiments, CTL were preincubated with various concentrations of anti-CD3 (Leu4),anti-CD8 (Leu2a), anti-CD4 (Leu3a), anti-CD56 (Leu19), anti-TCR α/β (WT31) or mouse IgG (isotype control), all from Becton Dickinson, Mountain View, CA for 30 min at 37°C before their addition to ^{51}Cr-labeled target cells. In some cases, target cells were incubated with anti-HLA class I mAb (W6/32, provided by Dr. Olivera Finn, Pittsburgh Cancer Institute); anti-HLA-DR mAb (obtained from Dr. Massimo Trucco, Pittsburgh Cancer Institute); antibodies against SCC-associated Ags, A9(22) and E7, kindly donated by Dr. Thomas Carey, University of Michigan (23), or E48, U36, K928, K984 or K931 antibodies kindly provided by Professor Gordon Snow, Free University, Amsterdam, The Netherlands (24-26), before cytotoxicity assays. Inhibition was calculated as:

$$\% \text{ inhibition} = 1 - \frac{\% \text{ specific lysis in mAb-treated wells}}{\% \text{ specific lysis in control wells}} \times 100$$

In some experiments, PCI-50 cells were incubated with 1000 U/ml IFNγ (Biogen, Cambridge, MA) or 1000 U/ml TNFα (Knoll Pharmaceuticals, Whippany, NJ) for 72h prior to cytotoxicity assays to examine the effects of cytokines on target cell susceptibility to lysis by the CTL line.

Effects of Cytokines or Conditioned Media on Growth of the PCI-50 cell line. Effects of various cytokines on growth of the PCI-50 cell line were determined using a colorimetric MTT [3-(4, 5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] assay (27). Briefly, doubling dilutions of each cytokine, a combination of cytokines or conditioned media from CTL or A-NK cell cultures were obtained in a 96 well flat-bottom plate (Costar), using TCM as diluent. Tumor cells ($5x10^3$/well) were added to a final volume of 200 μl per well. The plate was incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. On day 3 of culture , 50 μl of MTT solution (0.5% w/v; Sigma) were added to each well, and the plates were incubated for 4h. Following incubation, supernatant (150 μl) was removed from each well and replaced with 150 μl of DMSO (dimethyl sulfoxide; Sigma) to dissolve the formazan crystals. The plates were placed on a shaker for 15 min and read in a plate reader (Dynatech Laboratories Inc., Chantilly, VA),using a wavelength of 570 nm. Results from four wells for each dilution of the cytokines tested were expressed as percent control $\pm$ SD.

Flow cytometry. The phenotype of fresh or cultured lymphocytes was determined by two-color flow cytometry as described by us previously (2). Briefly, cells were adjusted to a concentration of $1x10^6$ /ml in PBS-0.1% (v/v) sodium azide solution, and 0.2 ml of this cell suspension was incubated with 5 μl of fluorescein- or phycoerythrin-labeled mAbs at 4°C for 30 min. The cells were then washed three times in PBS-sodium azide and fixed in 2% (w/v) paraformaldehyde solution in PBS. Two-color analysis was performed on FACScan (Becton Dickinson). The mAbs used were purchased from Becton Dickinson and included the following specificities: Leu4 (anti-CD3), Leu2a (anti-CD8), Leu3a (anti-CD4), Leu19 (anti-CD56), Leu15

(anti-CD11b), anti-IL2Rα (anti-CD25), anti-IL2Rß (p75), anti-HLA/DR, anti-TCR α/ß. As controls, mouse isotypes IgG1 and IgG2 were used in all experiments. The mAb to p75 IL2R was purchased from Endogen.

In addition, expression of HLA Ags, ICAM-1 or SCC-associated Ags on PCI-50 cells was examined. The tumor cell suspensions ($2x10^5$/tube) were first incubated with 5 μl of anti-HLA class I , or several concentrations of one of the following mAbs: E7, A9, E48, U36, K928, K984 or K931 at 4°C for 30 min, washed, and then incubated with fluorescein-labeled goat anti-mouse IgG (TAGO, Burlingame, CA) at 4°C for 30 min. Fluorescein-conjugated anti-ICAM-1 (AMAC, Westbrook, MA) and anti-HLA DR mAbs (Becton Dickinson) were also used in this study.

Proliferation assay. Proliferative responses of the CTL line were examined by co-culturing 10^4 responder cells with irradiated (8000 R) autologous PCI-50 cells (at responder:stimulator ratios which ranged from 1:1 to 1:8), allogeneic tumor cells or normal keratinocytes in 96-well round-bottomed plates at 37°C for 3 days in AIM-V medium supplemented with 300 IU/ml of IL2 and 300 U/ml of IL4. The cells were pulsed with 1 μCi of [^{3}H]-thymidine 16h before cell harvest and ^{3}[H]-thymidine incorporation was measured.

Statistical analysis. The significance of differences between experimental and control groups was analyzed using Student's t test or Wilcoxon's signed rank test, as appropriate. The level of significance was set at $p<0.05$.

RESULTS

Establishment and characteristics of the PCI-50 cell line. PCI-50 cell line was established from fresh SCCHN tumor explants. Once established, the tumor cell line grew rapidly (doubling time, 27.3h). In culture, the PCI-50 line grew as sharply demarcated, compact islands of cells with a distinct epithelial morphology (Fig.1a). The conditioned medium of the cell line contained small amounts of prostaglandin E_2 (PGE_2 up to 6 pg/10^4 cells) and almost

Fig.1a

no TGFß (up to 0.03 pg/10^4 cells). Histologic examination of H & E stained sections obtained from the patient's tumor showed a well-differentiated SCC, containing numerous keratinized epithelial pearls (Fig.1b). The tumor line was tumorigenic in nude mice. Following a subcutaneous injection of tumor cells ($5x10^6$ per mouse), tumors (7-10 mm in diameter, V=250-300 mm^3) developed within 4 weeks. On the other hand, these tumors grew subcutaneously in nude mice and had a poorly-differentiated morphology (Fig.1c). Fig.1b Fig.1c

Generation of the CTL lines reactive to PCI-50. To induce AuTu-reactive effector cells, irradiated (5000 R) or viable tumor cells were co-incubated with PBL obtained from the patient at the ratio of 5:1 in AIM-V medium in the presence of 300 IU/ml IL2 and 300 U/ml IL4. The PBL obtained prior to surgery or those obtained at two times after surgery and cultured with irradiated PCI-50 cells generally stopped growing by week 5. Cryopreserved fresh TIL did not proliferate at all. In, contrast, PBL co-cultured with non-irradiated viable tumor cell monolayers grew exponentially, and their cytotoxicity against AuTu continued to increase. Initially, these PBL had high levels of cytotoxicity against K562 or Daudi and showed no cytotoxicity against PCI-50 targets (Fig.2a). By week 6 in culture, the effector cells no longer lysed K562 or Daudi targets but had substantial anti-PCI-50 cytotoxicity. The CTL line lysed PCI-50 cells (e.g., 1,295 $LU_{20}/10^7$ cells, at week 10) and was maintained in long-term culture for over 20 weeks in the presence of viable tumor cells, IL2 and IL4. As shown in Fig.2b, by week 10-12 of culture, nearly all proliferating cells were $CD3^+DR^+$ and $CD8^+$, while the proportion of $CD4^+$ T cells was less than 10%. However, by week 20, the CD4/CD8 ratio of cultured cells shifted to about 1, and the CTL lost cytotoxic activity. When the same patient's PBL were obtained at 18 and 20 months after glossectomy, CTL lines again were generated, with reactivity against PCI-50 AuTu. These other CTL lines were similarly studied for phenotypic and functional characteristics and found to be identical to the first CTL line. These CTL lines also retained cytotoxic activity against PCI-50 targets for about 12 weeks in culture. No sign of tumor recurrence was observed in the patient during 2 years after surgery. This is of Fig.2a Fig.2b

interest, since it indicates that CTL precursors (CTL-p) for autologous SCCHN were present in the blood for at least 20 months in the absence of all detectable tumor. In mixed cultures containing tumor cells and CTL, rosettes of CTL surrounding the tumor cells were consistently observed at 24 hours after tumor stimulation (Fig.3). The presence of rosettes indicated that lymphoid cells were able to bind to AuTu cells in culture. Fig.3

Phenotypic and functional characteristics of the CTL line. To determine phenotypic properties of the CTL lines, flow cytometry studies were performed at various times in culture. As shown in Figure 2b, the culture established from PBL obtained before surgery contained more than 90% of $CD3^+CD8^+$ T cells by week 6. At week 8, the phenotype of the CTL line was $CD3^+$ (99%), $CD8^+CD11b^-$ (90%), TCR α/β^+ (93%), HLA-DR (90%), $CD56^+$ (72%), $CD25^+$ (52%), and IL2R $p75^+$ (93%). The presence of IL2R on SCCHN cell lines and normal keratinocytes was described by us earlier (28). In contrast to many other SCCHN cell lines, which usually express low levels of CD25 (28), the level of expression of CD25 (the IL2R-α chain) on PCI-50 was considerably greater, approaching that seen on keratinocytes in primary culture (28). The CTL lines established subsequently (i.e., from PBL obtained at 18 and 20 months after surgery) also had the same phenotype at week 8 in culture (data not shown).

The original CTL line was tested in 4h ^{51}Cr-release assays against SCCHN cell lines (n=4) and a variety of non-squamous human tumor (n=5) and normal (n=5) cell targets but was found to lyse only autologous SCCHN cell line (PCI-50, 482-1,295 $LU_{20}/10^7$ cells) and three allogeneic SCCHN (PCI-1, -2, -4B,:219, 132, or 141 $LU_{20}/10^7$ cells, respectively, see Figure 4). Fig.4 The CTL did not lyse allogeneic normal keratinocytes (n=3), fibroblasts (n=2), or a variety of non-squamous cell lines tested: gastric carcinoma (HR), 2 cholangiocarcinomas (SW and LP), K562 or Daudi. Thus, our data suggest that this CTL line probably recognizes a SCC-associated Ag(s) expressed on autologous and allogeneic SCCHN lines.

In order to better define cytotoxicity of AuTu by the CTL line, blocking experiments with mAbs were performed. Incubation of CTL with mAbs specific for the TCR α/β, CD3, CD8

(Fig.5a) or HLA class I Ags (Fig.5b) resulted in dose-dependent inhibition of cytotoxicity Fig.5a
against PCI-50, and similar results were obtained with PCI-1 target cells (data not shown). In
contrast, mAbs to CD4 or CD56 (Fig.5a) or to HLA-DR or SCC-associated Ags (A9 or E7) did
not block AuTu cytotoxicity (Fig.5b). Moreover, five additional mAbs to SCC-associated Ags Fig.5b
(E48, U36, K928, K984 or K931) also did not inhibit cytotoxicity of the CTL against PCI-50
(data not shown). These results indicate that recognition of an unknown Ag on autologous and
some allogeneic tumor cells by CTL was MHC-class I- restricted and involved the CD3-TCR
complex. HLA typing indicated that PCI-50 shared B44, DR4, DQW3 Ags with PCI-4 (A and
B refer to cell lines established from the primary and recurrent tumor, respectively).
Unfortunately, no HLA typing data were available for PCI-1 or PCI-2 cell lines. Studies are
in progress with additional cell lines to confirm the HLA-restricted nature of the CTL line-
SCCHN cell interaction.

To determine whether the CTL lines also proliferated in response to tumor cells,
[^{3}H]-thymidine incorporation tests were performed. As shown in Figure 6, the CTL line Fig.6
established from PBL obtained prior to surgery responded by dose-dependent proliferation to
PCI-50 (11,962 $\pm$ 1,742 cpm) and PCI-1 (5,418 $\pm$ 735 cpm) but not to epithelial gastric cancer cell
line, HR (1,544$\pm$166 cpm), or to normal keratinocytes (1,642 $\pm$ 152 cpm). The CTL had a
considerably higher level of response to autologous than to allogeneic SCCHN line.

Effects of cytokines on susceptibility of PCI-50 targets to CTL or their supernatants.
SCCHN cell lines have been shown by us earlier to be NK-cell resistant but sensitive to
lymphokine-activated killer (LAK) cells in 4h ^{51}Cr-release assays (27). The data presented in
Table 1 demonstrate that PCI-50 targets were resistant to resting NK cells (normal MNC), but Table 1
very sensitive to IL2-activated adherent NK (A-NK) cells and that the CTL lines we established
selectively lysed autologous tumor but not K562 or Daudi targets. In our previous experiments,
we observed that lysis of SCCHN targets by non-MHC-restricted effector cells was often
increased in vitro by preincubation of the tumor cells with exogenous cytokines (5). To

determine in vitro effects of various cytokines on PCI-50 susceptibility to lysis by CTL lines, we next preincubated these targets with various cytokines or mixtures of cytokines before cytotoxicity assays. As shown in Table 2, preincubation of PCI-50 targets with 1,000 U/ml of IFNγ, 1,000 U/ml of TNFα or a combination of the two cytokines for 3 days prior to ^{51}Cr-release assays significantly increased ($p<0.05$) lysis of PCI-50 targets by the CTL. Such preincubation was shown to be associated with increased expression of certain surface molecules on PCI-50 (Fig.7). While IFNγ significantly increased expression of class I-MHC Ags and ICAM-1, TNFα primarily upregulated expression of class I and class II MHC Ags (Fig.7). A combination of these two cytokines increased expression of all three types of surface Ags; however, an additive effect was observed only for class II MHC molecules (Fig.7). Pretreatment of PCI-50 with IFNγ was consistently more effective in augmenting lysis of PCI-50 targets by CTL line than TNFα (Table 2). Neither the two cytokines nor their combination increased expression of SCC-associated Ags, E7 or A9, on PCI-50 targets (data not shown).

Table 2

Fig.7

In addition to increasing tumor cell sensitivity to lysis by immune effector cells, cytokines have been shown to mediate cytostatic effects on SCCHN cell lines (7). PCI-50 cell line was not inhibited in growth by IFNγ, low concentrations (<2,000 U/ml) of TNFα or supernatants of the CTL lines (Fig.8). Although the tumor cells expressed the IL2Rα and IL2Rß, IL2 did not inhibit their growth over a wide range of concentrations tested (data not shown), as demonstrated by us for other SCCHN targets (28). However, when a combination of IFNγ and IL2 (22 nM) or supernatants of A-NK cells, known to contain significant levels of IFNγ and IL2 (5) were tested, growth of PCI-50 was significantly inhibited (Fig.8a and c). Similarly, high concentrations of TNFα had profound growth-inhibitory effects on PCI-50 cells in vitro.

Fig.8a-c

These experiments indicated that preincubation of PCI-50 targets with combination cytokines or certain effector cell supernatants could either alter the tumor cell sensitivity to lysis, inhibit tumor growth or both. Also, direct interaction of the CTL line with autologous

tumor cells appeared to be necessary for its antitumor activity, and CTL supernatants were not cytostatic or cytotoxic for AuTu in vitro.

DISCUSSION

Hallmarks of a T cell response to, e.g., tumor-associated Ag(s) (TAA) are its specificity and immunologic memory. The cytotoxic $CD8^+$ T cell recognizes Ags through the T-cell receptor (TCR) in association with a restriction element on the HLA class I peptide (29). The result of such recognition is a signal for activation, cytokine production, and expansion of a T cell clone, with concomitant generation of memory T lymphocytes (30). It has been uncertain to what extent Ags on human solid tumors are immunogenic for inducing specific CTL responses. Although CTL with "specificity" for AuTu have been obtained from peripheral blood or TIL in some patients with solid tumors, in most patients, it has not been possible to document the presence of such T cells in the periphery or at the tumor site. Also, rigorous studies to confirm AuTu specificity of CTL are difficult in humans, as AuTu cells, large panels of allogeneic tumor cells as well as normal cell targets are not readily available. It has been suggested that quantitative rather than qualitative antigenic changes, which occur on the surface of tumor cells, may not be recognized as "non-self" by the immune system (31). For this reason, in vivo development of CTL with AuTu specificity may be a rare event. Also, some tumors may produce immunosuppressive factors (32), while others may be associated with a defective Ag-presenting pathway (33), thus preventing generation of effective immune responses. In the case of SCCHN, humoral responses to TAA have been demonstrated and studied extensively (22-26). Monoclonal antibodies to TAA have been produced, and in some cases, these mAbs have been shown to recognize a unique Ag (e.g., K931) not present on normal squamous epithelium. In contrast, specific T-cell mediated responses to autologous SCCHN have been difficult to demonstrate, although a large body of evidence exists for the ability of these tumors to induce non-MHC-restricted effector mechanisms both in vivo and in vitro (2,4).

In this manuscript, we have described the generation and properties of AuTu-reactive CTL lines obtained from PBL of a patient with SCC of the tongue. The one intriguing aspect of the process of CTL generation has been that all three attempts at establishing a CTL line from this patient's PBL over a period of 20 months have been successful. In contrast, we have failed to establish CTL from PBL of 10 other individuals with SCCHN for whom AuTu cell lines and cryopreserved PBL were available, utilizing the same experimental conditions. These individuals either have recurrent disease or have died of disease. The ability to repeatedly generate CTL line from this patient's PBL indicates that CTL precursors (CTL-p) have been present in the peripheral circulation of this individual, and that in the presence of the AuTu cell line, these CTL-p have been able to proliferate and develop into cytotoxic effector cells. The patient, who was surgically treated, has not relapsed in more than 2 years following surgery and remains in good health in spite of an advanced age of 92 years. Clearly, this patient has had a strong antitumor immune response. In contrast, we were unable to establish a CTL line from cryopreserved TIL obtained from this patient's tumor at the time of surgery. This might be due to unfavorable in vitro conditions including considerable contamination of TIL with AuTu cells or production by the tumor of immunoinhibitory factors responsible for poor TIL proliferation in vitro. Even though PCI-50 supernatants contained only low levels of PGE_2 and no TGFß, other immunoinhibitory factors might down-regulate TIL response to IL2 (34). Also, levels of immunoinhibitory factors produced in vitro by PCI-50 line might not reflect levels of these factors produced in vivo.

The nature of an Ag(s) responsible for the TCR-mediated recognition of AuTu cells by our CTL lines is unknown. We have attempted to use a selection of available anti-SCC mAbs for inhibition of AuTu cell lysis by the CTL to obtain information about the Ag(s) the CTL recognize. However, none of these mAbs, e.g., to A9 integrin $\alpha^6ß_4$ (22) or to the E7 Ag, whose expression on tumor cells may be related to the chromosome 11 rearrangement in SCC (23), were able to block lysis of AuTu, PCI-50, by the CTL lines. A series of mAbs, E48, U36, K928, or

K984, which recognize squamous cell epithelial Ags on both normal and tumor epithelia (24-26) were also tested and found ineffective in blocking experiments. Because the CTL lines had strong lytic activity against AuTu and 3 allogeneic SCCHN, but not against other carcinomas tested so far or normal keratinocytes, it appears that the CTL recognize an Ag whose distribution is restricted to some SCCHN cell lines. Such a restricted distribution of the antigenic epitope, which presumably is recognized by the TCR in association with a class I MHC Ag, suggests that it is a common epitope shared by certain SCCHN but not by normal epithelial cells. Studies are in progress to determine both the identity of the MHC restricting element and biochemical nature of this Ag.

Although interactions between AuTu and CTL are dependent on the TCR-mediated recognition of a putative tumor Ag, other surface molecules appear to be involved as well. For example, preincubation of PCI-50 targets with TNFα or IFNγ increased expression of HLA-class I, HLA-class II and ICAM-I but not the SCC-associated Ags, E7 or A9. At the same time, such preincubation with cytokines also significantly increased susceptibility of AuTu to lysis by the CTL line. Thus, the addition of cytokines to the tumor in vitro and their ability to modulate expression of surface molecules on tumor target cells clearly contributes to antitumor effects mediated by the CTL. In addition to augmenting sensitivity of tumor cells to lysis by CTL, cytokines may also mediate cytostatic effects, and in the case of PCI-50, a combination of IFNγ and IL2 as well as TNFα at concentrations higher than 1000 U/ml were able to inhibit tumor growth in vitro. In contrast to culture supernatants of human allogeneic NK cells, supernatants of the CTL were not growth inhibitory for PCI-50 cell line. This observation suggests that direct contact of the CTL with AuTu targets is essential for activation of CTL and their antitumor effects, as also demonstrated by both rosette formation and proliferation in the presence of AuTu. Our preliminary observations regarding in vitro effects of cytokines and cellular supernatants of effector cells on growth or susceptibility to lysis of PCI-50 targets provide a basis for further studies of these effects in vivo.

Previous results from our laboratory indicated that immunotherapy of SCCHN with effector cells and/or cytokines such as IL2 or IFNγ or supernatants of A-NK cells may be effective in the control of tumor growth and lead to regression of established tumor in a xenograft model of SCCHN in nude mice (5,7,8). The availability of the AuTu cell line, PCI-50, as well as specific (the CTL lines) and non-specific (A-NK cells) effector cells allows us to compare their antitumor activities first in vitro and later in vivo, in the xenograft model of SCCHN in nude mice established in our laboratory (5,7,8). This combination of well-characterized effector cells should facilitate further studies of the mechanisms involved in effector-tumor target interactions and possibly lead to identification of tumor Ags which initiate and sustain these interactions. Understanding of these mechanisms is important for future development of novel therapeutic approaches for SCCHN, a relatively common cancer with significant morbidity and very high rates of recurrence (35) when treated with conventional therapy.

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Table 1. Lysis of the SCCHN cell line (PCI-50)

by immune effector cells[a]

Target cells	Effector cells		
	Resting MNC	A-NK cells	CTL line[b]
K562	153 ± 72	5863 ± 872	4 ± 4
Daudi	9 ± 3	3142 ± 456	1 ± 1
PCI-50	9 ± 4	2369 ± 580	1125 ± 228

[a] Cytotoxicity data (4h ^{51}Cr-release assays) are means ± SD of lytic units ($LU_{20}/10^7$ effector cells) from three experiments.

[b] The CTL line tested was established from PBL obtained prior to surgery.

Table 2. Effects of cytokines on susceptibility of PCI-50 SCCHN cell line to lysis by the CTL line

Treatment	Lytic units ($LU_{20}/10^7$ cells)[b]
None	853 ± 31
TNFα (1000 U/ml)	1096 ± 130*
IFNγ (1000 U/ml)	1624 ± 219*
TNFα + IFNγ	1266 ± 254*

[a] PCI-50 cells were incubated with medium or cytokines for 3 days prior to cytotoxicity assays. Tumor cells were washed and used as targets in cytotoxicity assays.

[b] Cytotoxicity data (4h ^{51}Cr-release assay) are means ± SD from three experiments. The CTL line established from PBL obtained before surgery was used.

* $p<0.05$, statistically significant difference in cytotoxicity of PCI-50 target incubated with or without cytokines.

LEGENDS

Figure 1. (A) Microscopic characteristics of PCI-50 line, which grows as a compact monolayer, Mag x 315; (B) histologic sections of the original tumor removed at the time of surgery and stained with hematoxylin & eosin (H&E) Mag x 315; (C) histologic sections of the tumor established by a subcutaneous injection of PCI-50 cell line derived from this human tumor (H&E) Mag x 315.

Figure 2. Changes in cytotoxic activities against K562 (—□—), Daudi (—▲—) or PCI-50 cells (—○—) of PBL co-cultured with AuTu in the presence of IL2 and IL4 (A). In (B), changes in the phenotype during culture of these PBL are shown. PBL were obtained from the patient before surgery.

Figure 3. Rosette formation between PCI-50 cells and the CTL line established from PBL obtained from the patient before surgery. Rosettes were usually observed within 24-48h of co-culture of the CTL with a viable PCI-50 monolayer. Rosettes shown were photographed after 48h of co-culture. Mag x 630.

Figure 4. Cytotoxicity of the AuTu-reactive CTL line (10-week culture). Cytotoxicity against a panel of tumor or normal cell targets was examined in 4h ^{51}Cr-release assays. The data are from one of three experiments performed.

Figure 5. Inhibition by various mAb of cytotoxicity mediated by the CTL line against PCI-50 targets. In (A), CTL were preincubated with 0.1-2 μg/ml of anti-CD3, anti-CD4, anti-CD8, anti-CD56, anti-TCR α/β, or isotype control antibodies for 30 min prior to 4h ^{51}Cr-release assays. In (B), PCI-50 cells were preincubated with diluted (final dilution, 2:1 to 16;1) anti-HLA class I, anti-HLA class II, or antibodies for the SCC-associated Ag (A9 or E7) for 30 min and then used as target cells in cytotoxicity assays. The data are from a representative experiment of three performed.

Figure 6. Proliferation of the CTL line in response to PCI-50 or allogeneic tumor cells. The CTL line ($1x10^4$/well) was incubated in the presence of IL2 and IL4 and irradiated autologous or allogeneic tumors for 3 days. The tumor cell:CTL ratios ranged from 1:8 to 1:1. ^{3}[H]thymidine incorporation was measured for 16h prior to harvest. An asterisk indicates a significant difference ($p<0.05$) compared with CTL in medium alone.

Figure 7. Expression of the HLA antigens, ICAM-1 and SCC-associated antigens on PCI-50 cells in the presence of cytokines. PCI-50 cells were incubated for 72h in the presence of medium, TNFα, IFNγ or both cytokines and then examined for expression of the HLA antigens, ICAM-1, A7 or E7 by flow cytometry. Abscissa, relative fluorescence intensity (log scale); ordinate, relative cell numbers (linear scale). The asterisks indicate a significant ($p<0.05$) shift in mean fluorescence intensity (MFI) from that in medium control.

Figure 8. Effects of IFNγ (—■—) or IFNγ plus IL2 (22 nM) (—●—) in (A), TNFα in (B), or supernatants of A-NK cells or CTL in (C) on growth of PCI-50 cells. Tumor cells ($5x10^3$/well) were cultured with various dilutions of cytokines or supernatants prepared in 96-well flat-bottom plates. On day 3 of culture, growth was measured in MTT assays. The data are presented as the percent of control ± SD obtained from triplicate wells. Shown are results of a representative experiment of 3 performed. The asterisks indicate a significant difference ($p<0.05$) compared with control cultures (medium alone).

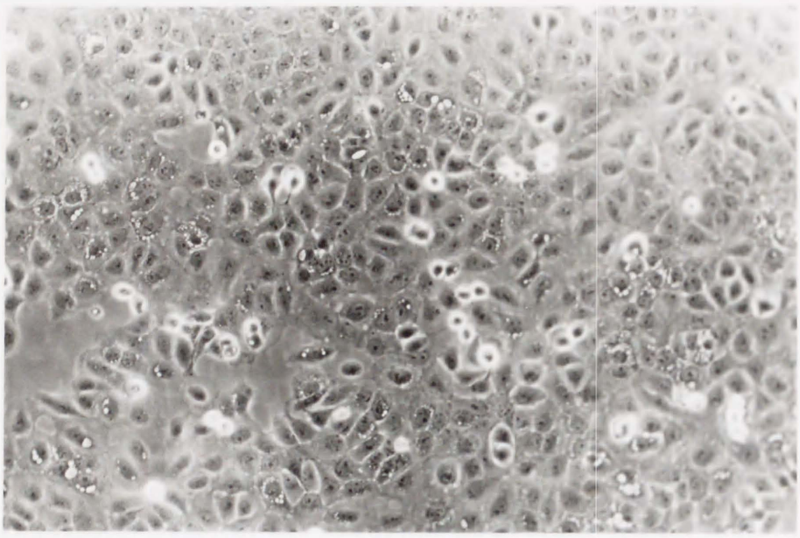


FIG.1 A

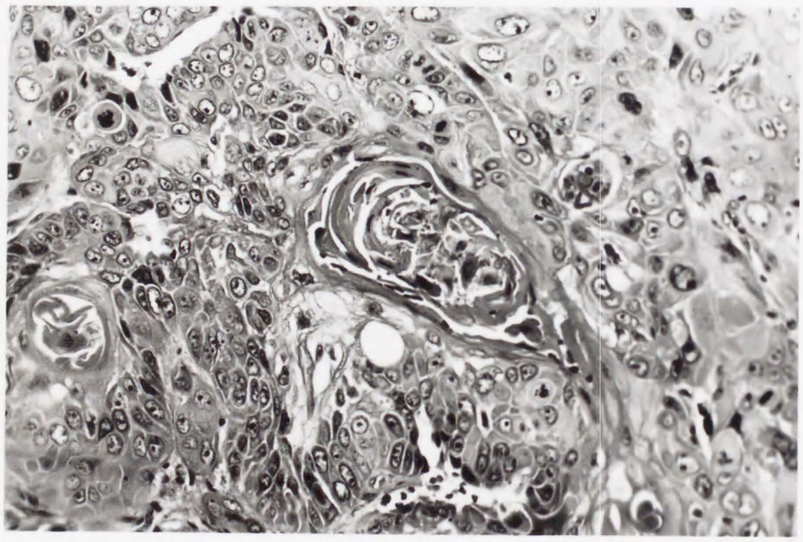


FIG.1 B

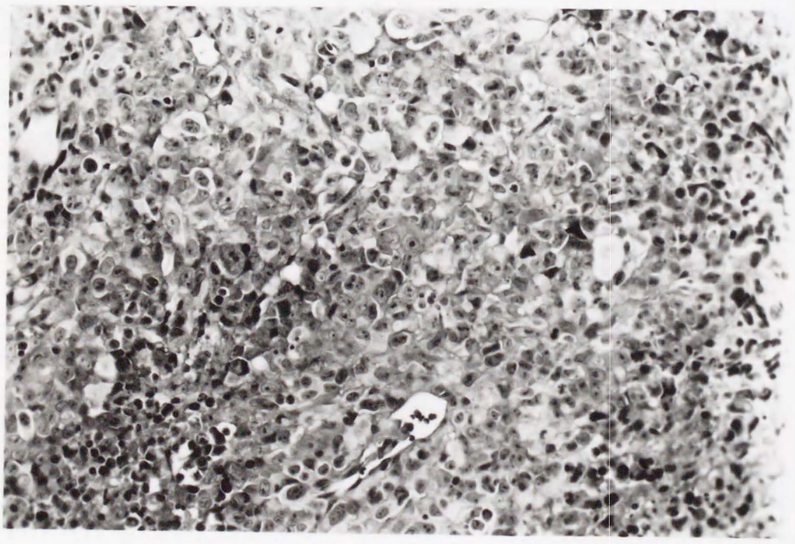


FIG.1 C

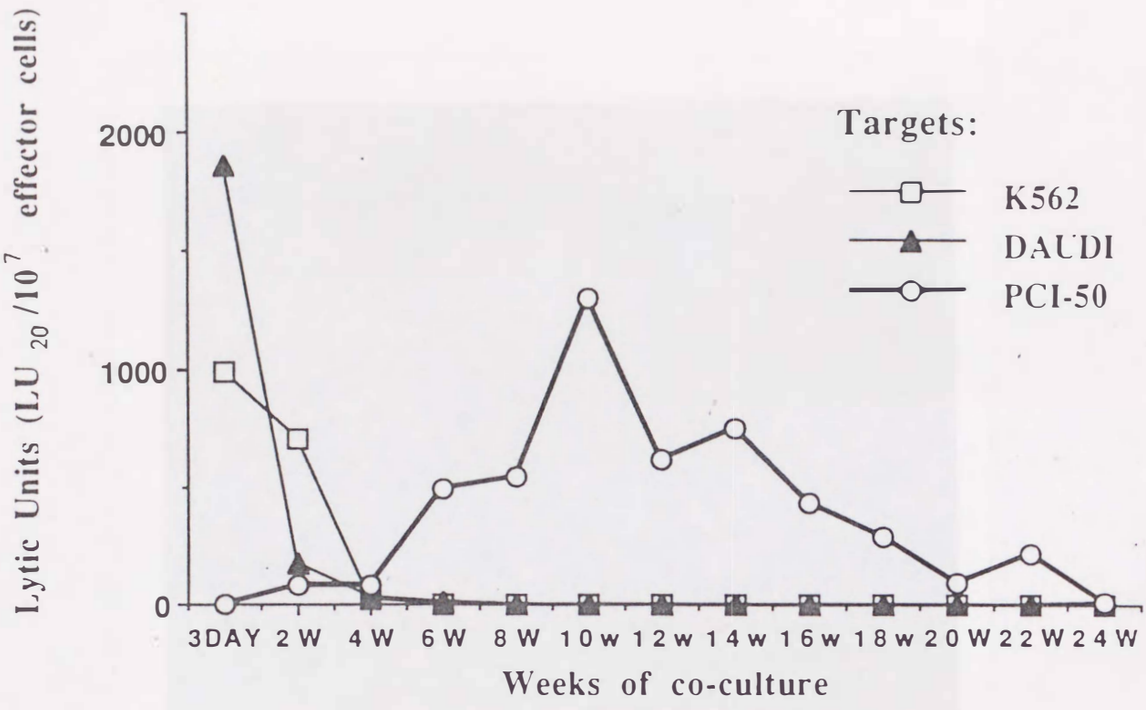

Lytic Units (LU 20 /10 7 effector cells)
2000
1000
0
Targets:
K562
DAUDI
PCI-50
3DAY 2W 4W 6W 8W 10w 12w 14w 16w 18w 20W 22W 24W
Weeks of co-culture

FIG. 2 A

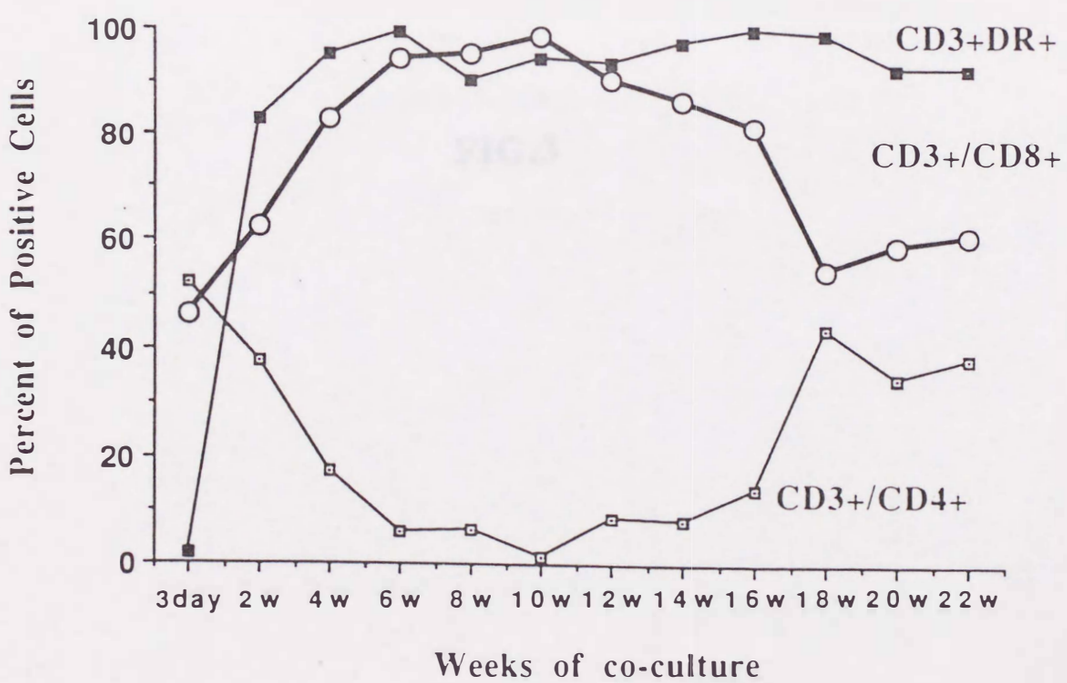

Percent of Positive Cells
100
80
60
40
20
0
CD3+DR+
CD3+/CD8+
CD3+/CD4+
3day 2w 4w 6w 8w 10w 12w 14w 16w 18w 20w 22w
Weeks of co-culture

FIG. 2 B

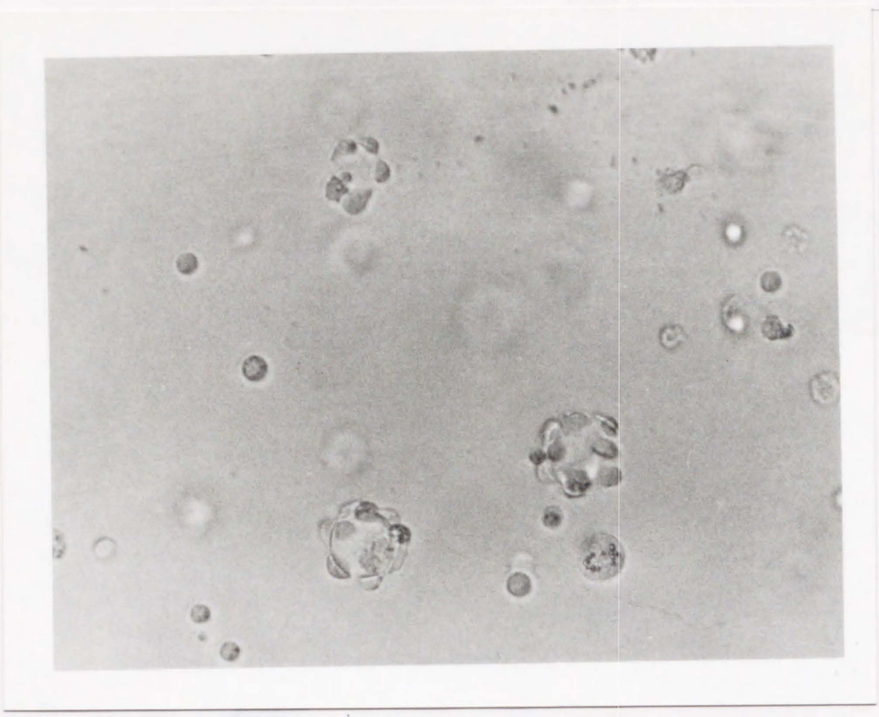


FIG.3

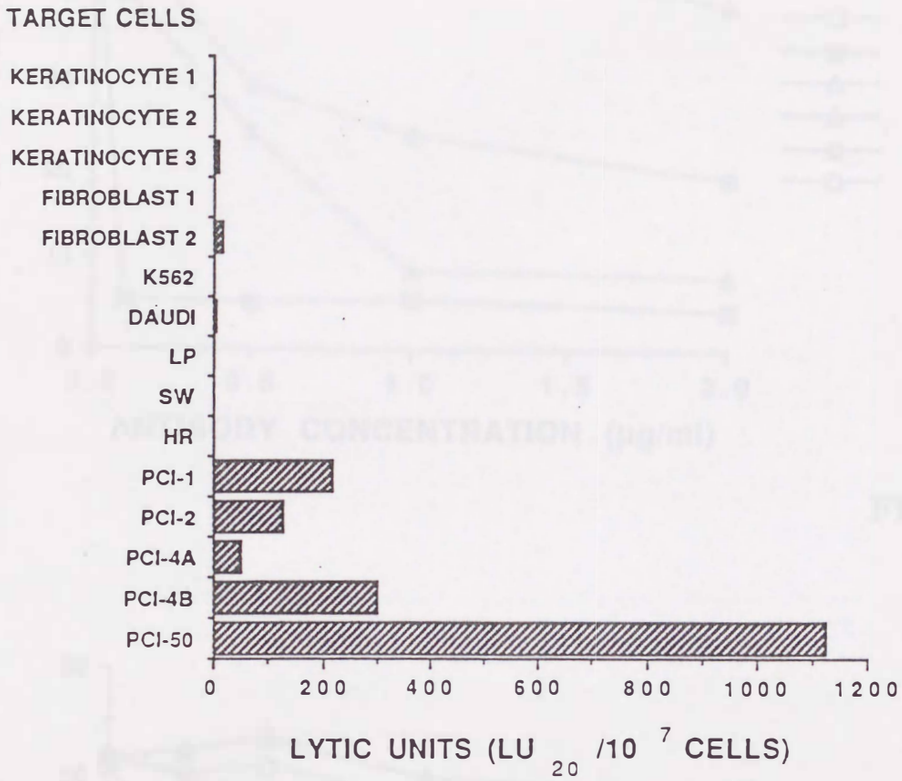

TARGET CELLS
KERATINOCYTE 1
KERATINOCYTE 2
KERATINOCYTE 3
FIBROBLAST 1
FIBROBLAST 2
K562
DAUDI
LP
SW
HR
PCI-1
PCI-2
PCI-4A
PCI-4B
PCI-50
0
200
400
600
800
1000
1200
LYTIC UNITS (LU 20 /10 7 CELLS)

FIG. 4

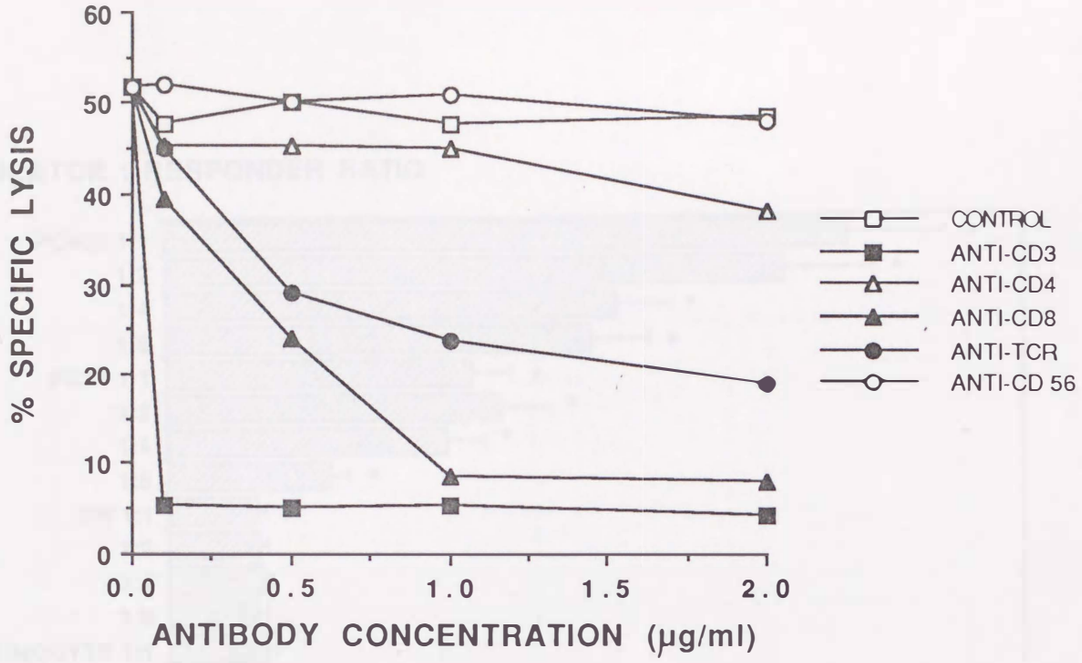

60
50
40
30
20
10
0
% SPECIFIC LYSIS
0.0
0.5
1.0
1.5
2.0
ANTIBODY CONCENTRATION (µg/ml)
CONTROL
ANTI-CD3
ANTI-CD4
ANTI-CD8
ANTI-TCR
ANTI-CD 56

FIG. 5 A

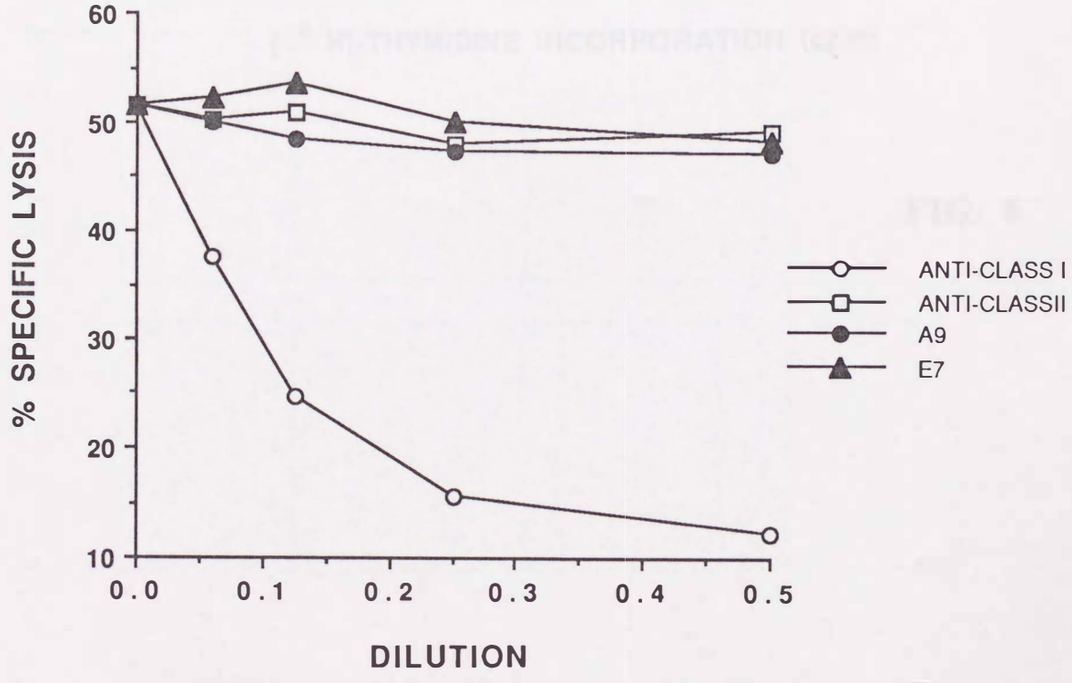

60
50
40
30
20
10
% SPECIFIC LYSIS
0.0
0.1
0.2
0.3
0.4
0.5
DILUTION
ANTI-CLASS I
ANTI-CLASSII
A9
E7

FIG. 5 B

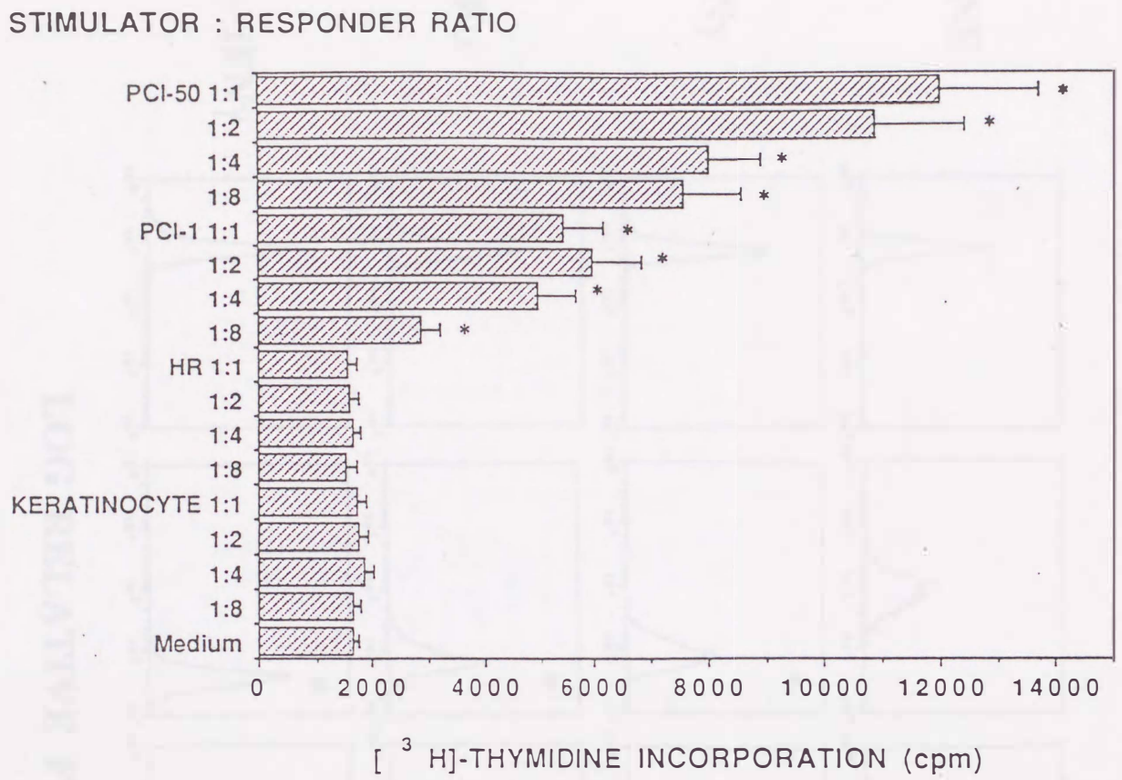

STIMULATOR : RESPONDER RATIO
PCI-50 1:1
1:2
1:4
1:8
PCI-1 1:1
1:2
1:4
1:8
HR 1:1
1:2
1:4
1:8
KERATINOCYTE 1:1
1:2
1:4
1:8
Medium
0
2000
4000
6000
8000
10000
12000
14000
[3 H]-THYMIDINE INCORPORATION (cpm)

FIG. 6

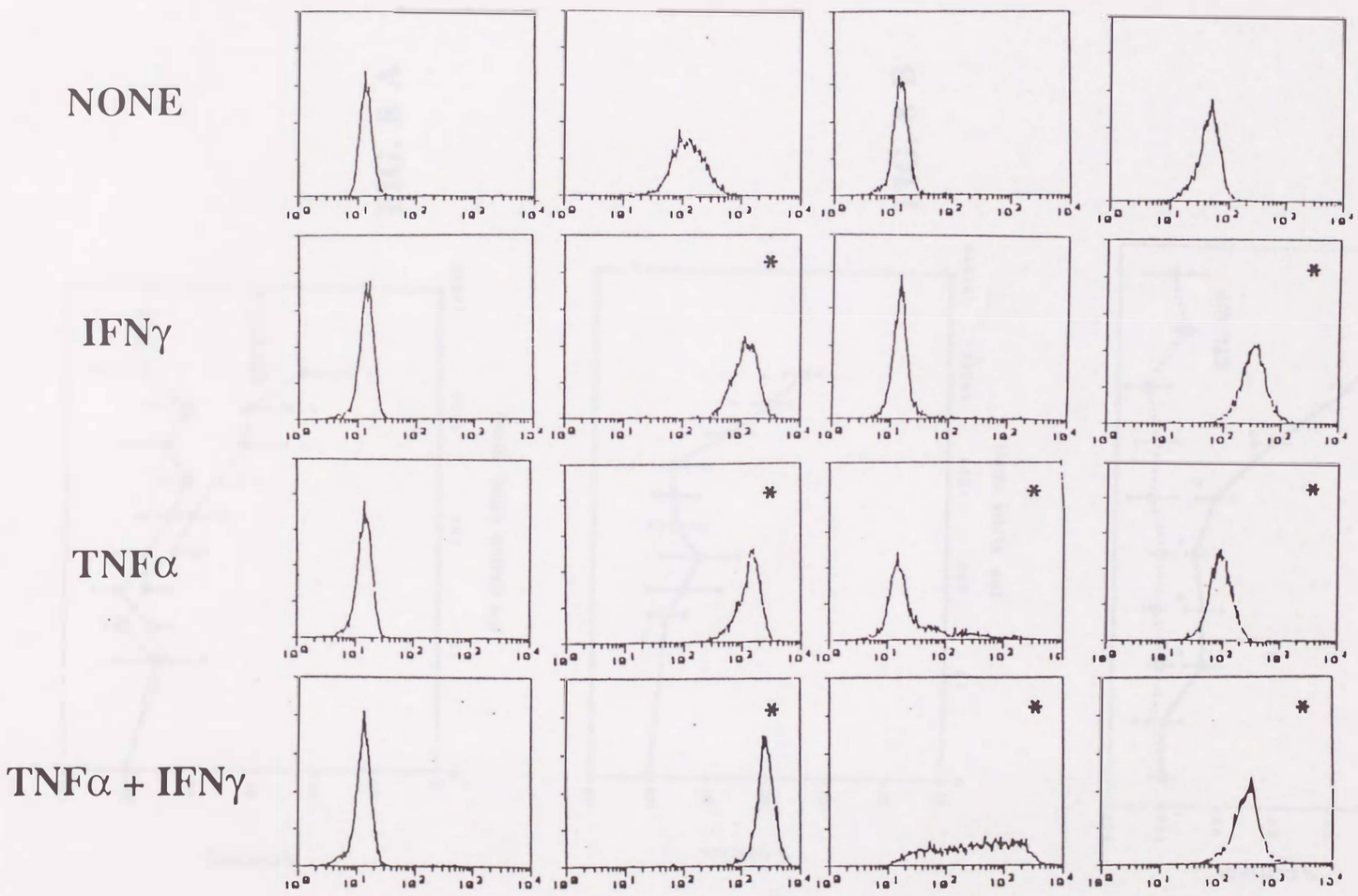

CYTOKINES
IgG1
CLASS I
DR
ICAM-1
NONE
IFNγ
TNFα
TNFα + IFNγ
*
RELATIVE CELL NUMBER
LOG RELATIVE FLUORESCENCE

FIG. 7

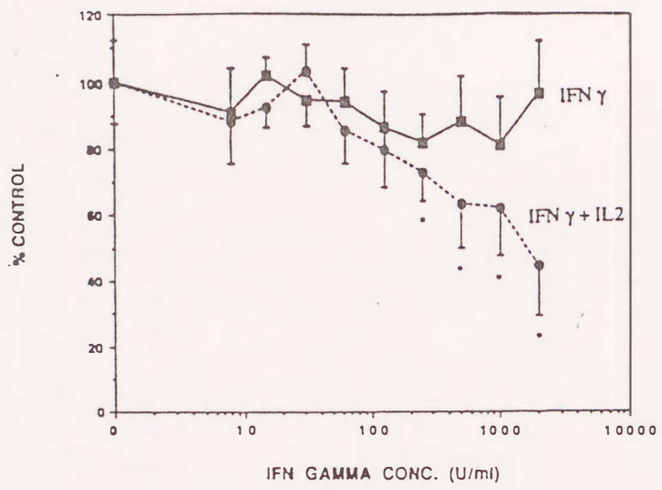


FIG. 8 A

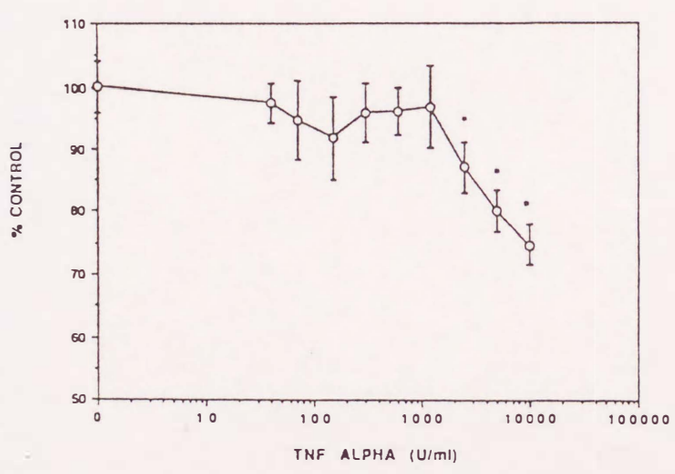


FIG. 8 B

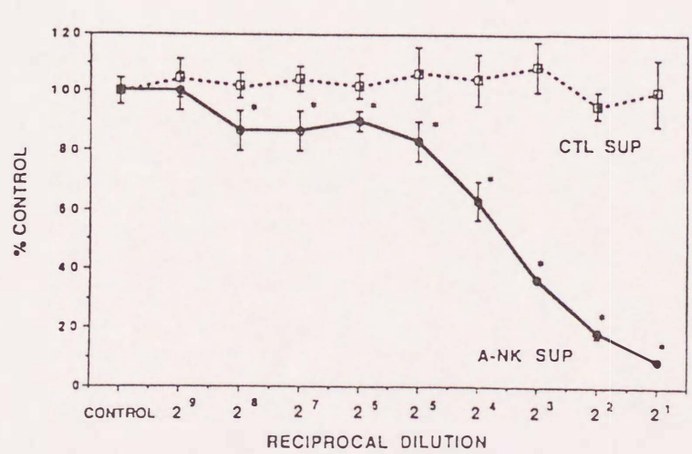
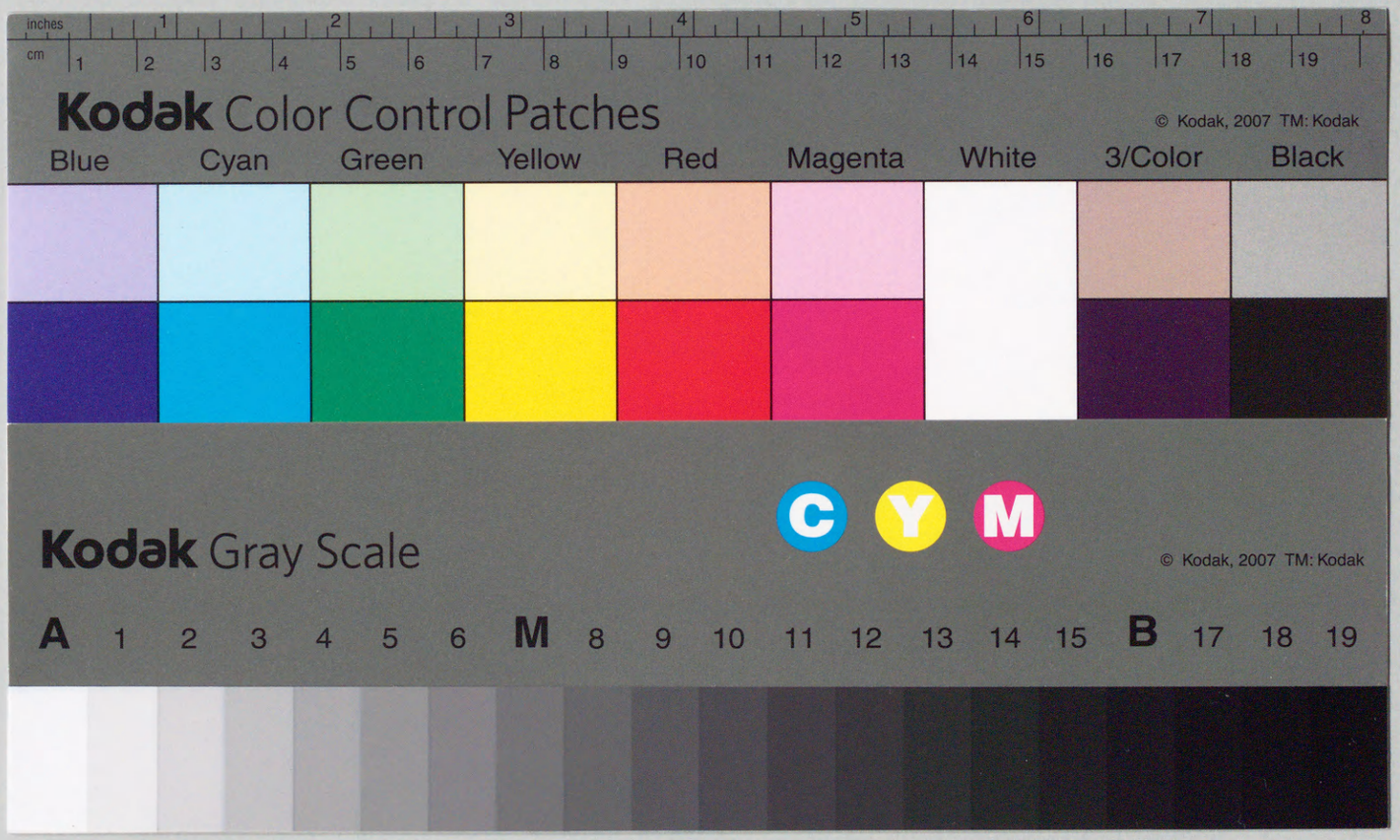


FIG. 8 C


inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19