Under the conditions suggested, triple conjugates of the R_A-DAD-IgG type can easily be obtained with a satisfactory yield (fig. 1). The R_A-DAD-L_1 conjugate preserves its ability to bind with the surface of SMM cells (indirect fluorescence measurements, data not shown), whereas the intermediate compounds R_A-DAD and R_A-DAD-IgG (MOPS) do not bind to SMM cells (see figs 2 and 3).

It was found that at a concentration of 10^{-6}–10^{-7} M the conjugate R_A-DAD-L_1 suppressed ^{14}C-leucine incorporation into SMM cells by 90% and practically did not influence ^{14}C-leucine incorporation into control IAR-2 cells. Pure native ricin at the same concentrations suppresses ^{14}C-leucine incorporation in both cell types by 90–95%. A nonspecific conjugate of the R_A-DAD-IgG (MOPC) type did not decrease protein synthesis either in SMM or in IAR-2 cells (figs 2, 3). Control use of pure R_A, native or oxidized dextran, specific and nonspecific IgG only slightly affect normal ^{14}C-leucine incorporation (protein synthesis) in both cell types; the maximal decrease observed was about 20% in the case of R_A.

Thus, only the R_A-DAD-L_1 conjugate specifically binds SMM cells and suppresses protein synthesis, causing cell death.

The data obtained show that L_1 antibodies can be used as vectors for targeted transport of different substances to the modulated smooth muscle cells. This is particularly helpful for the selective screening of proliferating smooth muscle cells in vascular pathology models in rats[16,17].

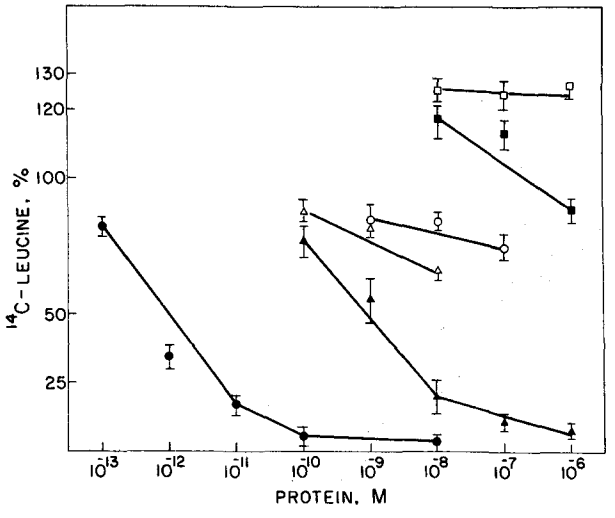

Figure 3. Cytotoxic effect on SMM cells of different concentrations of ricin (●), R_A (○), R_A-DAD-L_1 (▲), R_A-DAD-IgG (MOPC) (■), R_A-DAD (△), pure L_1 (□).

1 Schwartz, S.M., Proc. Soc. exp. Biol. Med. *173* (1983) 1.
2 Thomas, W.A., and Kim, D.M., Lab. Invest. *48* (1983) 245.
3 Chamley, J., Campbell, G., and Ross, R., Physiol. Rev. *59* (1979) 1.
4 Chamley, J., Campbell, G., and Ross, R., J. Cell Biol. *89* (1981) 379.
5 Printseva, O.Yu., Faerman, A.I., Tyurmin, A.V., and Ilynsky, O.B., Doklady AN SSSR (Russ.) *275* (1983) 2171.
6 Printseva, O.Yu., Faerman, A.I., Tyurmin, A.V., and Ilynsky, O.B., J. Cell Biol. *97* (1983) 1759.
7 Vitetta, E.S., Krolick, K.A., Miyama-Inaba, M., Cushley, W., and Uhr, J.W., Science *219* (1983) 644.
8 Tsukada, Y., Bischof, W.K.-D., Hibi, N., Hirai, H., Hurwitz, E., and Sela, M., Proc. natn. Acad. Sci. USA *79* (1982) 621.
9 Tsukada, Y., Hurwitz, E., Kashi, R., Sela, M., Hibi, N., Hara, A., and Hirai, H., Proc. natn. Acad. Sci. USA *79* (1982) 7896.
10 Ilynsky, O.B., Klueva, T.S., Krasnikova, T.L., Printseva, O.Yu., Solov'eva, A.I., and Tyurmin, A.V., Bull. exp. Biol. Med. (Russ.) *2* (1984) 183.
11 Nicolson, G.L., and Blunstein, J., Biochim. biophys. Acta *266* (1972) 543.
12 Olsnes, S., and Pihl, A., Biochemistry *12* (1973) 3121.
13 Anderson, C.W., Baum, P.R., and Gesteland, R., J. Virol. *12* (1973) 241.
14 Pelham, R., and Jackson, R., Eur. J. Biochem. *67* (1976) 247.
15 Torchilin, V.P., Reizer, I.L., Smirnov, V.N., and Chazov, E.I., Bioorgan. Khimia (Russ.) *2* (1976) 1252.
16 Boudjers, G., and Björnheden, T., Atherosclerosis *12* (1970) 301.
17 Koleisky, S., and Snajdar, R.M., Am. J. Path. *103* (1981) 105.

0014-4754/85/101342-03$1.50 + 0.20/0

Contrasting effects of RNA and protein synthesis blocking on natural and lectin-dependent cell-mediated cytotoxicity against adherent HEp-2 cells

A. Perl, R. Gonzalez-Cabello, L. Falucskai, P. Gergely and J. Fehér

Division of Immunology, Second Department of Medicine, Semmelweis University, Szentkirályi u. 46, H-1088 Budapest (Hungary), 6 August 1984

Summary. In this study, earlier observations[2,9] concerning the independence of both natural (NCMC) and lectin-dependent cell-mediated cytotoxicity (LDCC) from DNA synthesis have been confirmed. In addition, blocking of RNA synthesis by actinomycin D and of protein synthesis, reversibly by puromycin (PM) and irreversibly by emetine (EM) had different effects on NCMC and LDCC against ^{3}H-thymidine-prelabeled HEp-2 target cells. Similarly to the Con A-induced proliferation of lymphocytes, LDCC activity was also inhibited by blocking of RNA and protein synthesis. NCMC to HEp-2 target cells was not affected by blocking of RNA synthesis, while both PM and EM strongly enhanced NCMC activity.

Key words. Natural cell-mediated cytotoxicity; lectin-dependent cell-mediated cytotoxicity; RNA synthesis; protein synthesis.

The molecular mechanism of cell-mediated cytotoxicity has recently prompted extensive investigaitons[1,4]. In all types of in vitro cytotoxic reactions, such as specific T cell-mediated and nonspecific lectin-dependent (LDCC), oxidation-dependent (ODCC) as well as natural killer (NK) cell-mediated cytotoxicity, after binding of the effector lymphocyte to the target cell, the 'lethal blow' is thought to be delivered by the interaction of preformed cell surface structures[1,4] on the effector and target cells. Berke has suggested that one cytotoxic T cell receptor has two functions: binding to and lysis of the target cell[1], whereas Bonavida et al. reported that binding and lysis can be separately blocked by different specific antibodies[4]. We have earlier demonstrated profoundly depressed levels of LDCC in patients with systemic lupus erythematosus[10] and carcinoma of the uterine cervix[11], using a sensitive and reproducible LDCC assay, by detachment from the monolayer of ^{3}H-thymidine-prelabeled HEp-2 adherent target cells with concanavalin A (Con A). These observations have supported the putative role of endogeneous lectins and/or lectin-like receptors[8] in cytotoxicity changes observed in primary and secondary diseases of the immune system. In this cytotoxicity system the major effector cells are T lymphocytes. Both $OKT4^+$ and $OKT8^+$ T cell subsets and LDCC

effector activity against HEp-2 cells without having detectable cell surface HLA-A, B, C and D/DR antigens[14]. This implies that neither of these lymphocyte differentiation antigens nor MHC-coded proteins of target cells are a prerequisite for LDCC. Recently, we observed that natural cell-mediated cytotoxicity (NCMC) and LDCC against HEp-2 cells were affected in opposite ways by Fc-receptor blocking[12], as well as by the presence of carrageenan[13]. In the present study, to uncover possible differences in the basic effector mechanisms of NCMC and LDCC, were examined in parallel the effect of DNA, RNA and protein synthesis blocking on cytotoxicity against HEp-2 cells.

Materials and methods. Separation of peripheral blood mononuclear cells. Human peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood of healthy donors on a Ficoll-Uromiro gradient[5], and resuspended in TC 199 tissue culture medium supplemented with 10% heat-inactivated calf serum (Phylaxia, Hungary), 25 mM HEPES buffer (SERVA, GFR), 2 mM L-glutamine (GIBCO, USA), 100 IU/ml penicillin and 100 µm/ml gentamicin.

Target cells. Target cells derived from the Cincinnati HEp-2 adherent human epipharynx carcinoma cell line (National Institute for Public Health, Budapest, Hungary) were cultured in Eagle's MEM containing 10% heat-inactivated calf serum, 25 mM HEPES, 2 mM L-glutamine, 300 IU/ml penicillin, 120 µg/ml gentamicin and 7.5 µg/ml amphotericin B, in 100 ml sterile plastic tissue culture flasks (Greiner, FRG). Serial passage of HEp-2 cell monolayers was performed in 3–5 day periods. After discarding detached, dead cells with the supernatant medium, HEp-2 target cells for cytotoxicity assay were resuspended from the bottom of culture flasks with 0.5 ml of 1% trypsin (Sigma, USA) and 0.05% Versene (GIBCO, USA) in TC 199, then washed twice in culture medium. Viability of resuspended target cells was evaluated by trypan blue staining (> 99%).

Cytotoxicity assay. Resuspended HEp-2 target cells (2500 cells in 100 µl culture medium per well) were seeded in flat-bottomed microtiter plates (Greiner, FRG), then prelabeled with 0.4 µCi ^{3}H-thymidine (UVVR, Czechoslovakia, ^{3}H-TdR) in 20 µl culture medium. To allow cell adhesion the plates were incubated at 37 °C for 24 h in a humidified atmosphere with 5% CO_2. After 24 h HEp-2 cells grew to a monolayer, and incorporated sufficient isotope acitvity (2–2.5 × 10^4 cpm) for cytotoxicity studies. This number of target cells per well also allowed the addition of effector cells at a high ratio up to 50:1. Subsequently, each well was washed twice with 37 °C culture medium. The assays were performed in six replicates. To each well, 100 µl effector cell suspension was added in TC 199 at an optimal killer-to-target ratio of 50:1. LDCC cultures also contained Con A (Pharmacia, Sweden) at a final concentration of 25 µl/ml. Target cells incubated in medium alone, and in medium plus Con A but without effectors were included in each experiment. No target cell damage due to Con A was observed in the absence of added effector cells. Culture plates were incubated for 24 h at 37 °C. Detached target cells (dead according to trypan blue staining) and effector cells were removed by washing twice with 37 °C culture medium. Detachment from the monolayer was used as an indication of cell damage[6]. The remaining adherent HEp-2 cells were frozen at −20 °C. After thawing, the content of each well was sucked off on to filter paper discs by an automated sample harvester (Dynatech, FRG). Isotope determinations were made in a liquid scintillation counter (Nuclear Chicago Isocap 300, USA). The results were expressed in cpm using the arithmetic mean of six replicate values. Natural cell-mediated cytotoxicity (NCMC) was calculated taking the medium control as baseline, according to the formula:

$$\% \text{ cytotoxicity (NCMC)} = 100 - \frac{\text{cpm test sample}}{\text{cpm medium control}} \times 100.$$

LDCC was expressed as the percentage increase of cytotoxicity above NCMC attributable to the presence of Con A:

$$\% \text{ LDCC} = \left(100 - \frac{\text{cpm test sample with Con A}}{\text{cpm medium control with Con A}} \times 100\right) - \left(100 - \frac{\text{cpm test sample without Con A}}{\text{cpm medium control without Con A}} \times 100\right).$$

Proliferative responses to Con A. 2 × 10^5 PBMC suspended in TC 199, supplemented with 10% heat-inactivated calf serum, 25 mM HEPES, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml gentamicin and 7.5 µg/ml amphotericin B, were placed in each microplate well using four parallel samples. Con A was added in an optimal concentration of 25 µg/ml. Control cultures without Con A were included in each experiment. The plates were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 96 h. The cultures were pulsed with 0.4 µCi ^{3}H-TdR 8 h before termination. The cells were harvested on to filter paper discs as described above.

DNA, RNA and protein synthesis blocking. DNA synthesis of PBMC was blocked by pretreatment with 50 µg/ml (1.5 × 10^{-7} M) mitomycin C (Mc, Sigma, USA) for 30 min at 37 °C. After washing twice, PBMC were added to HEp-2 targets. Blocking of RNA synthesis was performed by pretreatment of PBMC with 0.025, 0.25 and 2.5 µg/ml (2 × 10^{-9} M) actinomycin D (AD, Serva, FRG) for 30 min at 37 °C. Subsequently, PBMC were washed twice and added to HEp-2 cells. Inhibition of protein synthesis by pretreatment of PBMC with up to 10^{-4} M puromycin (PM, Sigma) for 60 min at 37 °C proved to be reversible after

Table 1. Effect of DNA, RNA and protein synthesis blocking on NCMC and LDCC against HEp-2 cells

	% NCMC	p	% LDCC	p
Control	11 ± 6	–	40 ± 6	–
Mitomycin C (MC, 1.5 × 10^{-7} M)	+ 5 ± 5	NS	+ 2 ± 5	NS
Actinomycin D (AD, 2 × 10^{-9} M)	+ 0 ± 9	NS	− 28 ± 8	< 0.05
Duromycin (PM, 2 × 10^{-6} M)	+ 71 ± 15	< 0.01	− 30 ± 11	< 0.05
Emetine (EM, 10^{-6} M)	+ 10 ± 6	NS	− 9 ± 19	NS
(10^{-5} M)	+ 22 ± 5	< 0.05	− 14 ± 4	< 0.05
(10^{-4} M)	+ 57 ± 9	< 0.01	− 28 ± 7	< 0.01

NCMC was measured by detachment from the monolayer of 2500 ^{3}H-TdR prelabeled HEp-2 cells per well of microtiter plates, while % LDCC showed increase of cytotoxicity above NCMC due to 25 µg/ml concanavalin A. PBMC effector cells were pretreated with 1.5 × 10^{-7} M (50 µg/ml) MC, or with 2 × 10^{-9} M (2.5 µg/ml) AD, or with 10^{-6} to 10^{-4} M EM while 2 × 10^{-6} M PM was present during the cytotoxicity assay. Positive values show enhancement, negative ones inhibition of cytotoxicity as differences in comparison to control values. Data represent mean ± SEM of eight experiments in a 24-h assay at an effector:target cell ratio of 50:1; p-values refer to the effect of drug treatment using the paired t-test; NS, not significant.

Table 2. Effect of DNA, RNA and protein synthesis blocking on the proliferative response of PBMC to Con A

Reagent	Medium control	Test sample	p
Mitomycin C (MC, 1.5 × 10^{-7} M)	12,060 ± 1069	976 ± 97	< 0.01
Act (AD, 2 × 10^{-9} M)	16,643 ± 3786	199 ± 31	< 0.05
Puromycin (PM, 2 × 10^{-6} M)	27,460 ± 5128	383 ± 77	< 0.001
Emetine (EM, 10^{-4} M)	8420 ± 4478	155 ± 24	< 0.05

Proliferate response of 200,000 PBMC per microplate well was measured after incubation for 96 h at 37 °C with 25 µg/ml Con A. Control cultures without Con A were also included. The cultures were pulsed with 0.4 µCi ^{3}H-TdR 8 h before termination. Drug treatments were performed as described in table 1. Data represent mean ± SEM of six experiments. p-values refer to the effect of drug treatment using the paired t-test.

washing the cells twice measured by ^{3}H-leucine incorporation[2]. Protein synthesis of PBMC was effectively blocked in the presence of PM even at a low dose of 2×10^{-6} M, or by pretreatment of PBMC with emetine HCl (EM, Reanal, Hungary) for 45 min at 37 °C. After EM pretreatment PBMC were washed twice before addition to HEp-2 targets. At the doses applied neither of the drugs affected the viability of effector (trypan blue exclusion) or target cells (unchanged adherence).

Statistics. Data are given as a mean ± SEM of n experiments. Statistical were performed with Student's t-test.

Results and discussion. Blocking of DNA synthesis by MC pretreatment of PBMC had no major effect on either NCMC or LDCC to HEp-2 cells (table 1). Similar results were obtained using ^{51}Cr-labeled K 562 targets[2,9]. In parallel experiments, MC pretreatment completely abrogated Con A-induced lymphocyte proliferation (table 2).

Inhibition of NK activity against K 562 target cells was demonstrated by pretreatment of effector cells with actinomycin D[2]. The relationship of LDCC to RNA synthesis has not yet been investigated. Blocking of RNA synthesis by AD pretreatment of PBMC dose-dependently reduced both LDCC activity and Con A-induced blastogenesis. LDCC (table 1) and lymphocyte proliferation (table 2) were significantly inhibited by AD at a concentration of 2.5 µl/ml (2×10^{-9} M). AD had no major influence on NCMC against HEp-2 cells (table 1). This suggests that contrary to NCMC, LDCC against HEp-2 targets requires the synthesis of new RNA molecules, similar to the Con A-induced proliferation and the NK activity[2] of lymphocytes.

Recently, in the presence of a reversible protein synthesis blocker, puromycin (PM), NK activity against K 562 targets was inhibited but could be easily reversed by washing out the drug, while emetine HCl (EM) irreversible blocked both protein synthesis and NK activity against K 562 targets[2]. Sawada and Osawa have earlier demonstrated inhibition by PM of LDCC in the mouse[15]. Pretreatment of PBMC by PM, even at the high concentration of 10^{-4} M, failed to influence NCMC and LDCC activities, as well as Con A-induced blastogenesis (data not shown). In the presence of PM, even at a low dose of 2×10^{-6} M, a strong stimulation of NCMC ($p < 0.01$), and inhibition of LDCC ($p < 0.05$) occurred (table 1). PM also abrogated Con A-induced blastogenesis (table 2). Though PM in the dose applied effectively blocked protein synthesis, a direct effect of the drug on NCMC and LDCC could not be entirely ruled out. Thus, the relationship of NCMC and LDCC with protein synthesis was also studied using EM, an irreversible protein synthesis blocker[2]. As is shown in table 1, EM dose-dependently enhanced NCMC, and reduced LDCC, similarly to the changes observed in the presence of PM. In parallel experiments Con A-induced blastogenesis of PBMC was also blocked by EM pretreatment (table 2).

The present results confirm earlier observations that both NCMC and LDCC independent of DNA synthesis[2,9]. Furthermore, these data reveal a different relationship between RNA and protein synthesis as well as NCMC and LDCC, respectively. Likewise, the Con A-induced proliferation of lymphocyte LDCC activity also requires de novo RNA and protein synthesis, while NCMS against HEp-2 target cells depends on preformed structures. Very recently, cytotoxic macrophages were shown to perform down regulation of RNA labeling[18], and to have a requirement for protein synthesis upon stimulation by alpha- and beta-interferon but not by gamma-interferon[3]. In contrast to the NCMC against HEp-2 carcinoma cells NK activity towards K 562 leukemia target cells was inhibited by both RNA and protein synthesis blockers[2]. This can be associated with previous data suggesting the heterogeneity of cytotoxic mechanisms against carcinoma/sarcoma/solid tumor/ as well as leukemia/lymphoma target cells in the mouse[7,16]. Augmentation of NCMC by blocking of protein synthesis could be related to the blocking of the synthesis of an inhibitory protein for NCMC by PM and EM, or to effects of PM and EM on suppressor cells for NCMC[17]. This question is currently being investigated in our laboratory.

Acknowledgment. We thank Mrs Maria Schender for excellent technical assistance.

1 Berke, G., Immun. Rev. *72* (1983) 5.
2 Bishop, G.A., and Schwartz, S.A., Clin. Immun. Immunopath. *25* (1982) 374.
3 Blasi, E., Herberman, R.B., and Varesio, L., J. Immun. *132* (1984) 3226.
4 Bonavida, B., Bradley, T., Fan, J., Hiserodt, J., Effros, R., and Wexler, H., Immun. Rev. *72* (1983) 119.
5 Böyum, A., Scand. J. clin. Lab. Invest. *21* suppl. 97 (1968) 1.
6 Huges-Law, G., De Gast, G.C., and The, T.H., J. immun. Meth. *19* (1978) 29.
7 Lattime, E.C., Pecorado, G.A., and Stutman, O., J. exp. Med. *157* (1983) 1070.
8 MacDermott, R.P., Kienker, L.J., Bertovich, M.J., and Muchmore, A.V., Immunology *44* (1981) 143.
9 Masucci, G., Masucci, M.G., and Klein, E., Cell. Immun. *69* (1982) 21.
10 Perl, A., Gonzalez-Cabello, R., Láng, I., and Gergely, P., Immun. Comm. *11* (1982) 431.
11 Perl, A., Gonzalez-Cabello, R., Láng, I., Somos P., and Gergely, P., Cancer Immun. Immunother. *15* (1983) 155.
12 Perl, A., Gonzalez-Cabello, R., Pócsik, É., Láng, I., and Gergely, P., Immun. Lett. *6* (1983) 317.
13 Perl, A., Gonzalez-Cabello, R., and Gergely, P., Clin. exp. Immun. *54* (1983) 567.
14 Perl, A., Gonzalez-Cabello, R., Láng, I., and Gergely, P., Cell. Immun. 84 (1984) 185.
15 Sawada, J., and Osawa, T., Immunology *41* (1980) 525.
16 Stutman, O., Immun. Today *2* (1981) 205.
17 Tarkkanen, J., and Saksela, E., Scand. J. Immun. *15* (1982) 149.
18 Varesio, L., J. Immun. *132* (1984) 2683.

0014-4754/85/101344-03$1.50 + 0.20/0

Temperature-dependent responses to a developmental gradient in the *Drosophila* wing[1]

J. N. Thompson, Jr, W. E. Spivey and D. K. Duncan

Department of Zoology, University of Oklahoma, Norman (Oklahoma 73019, USA), 10 October 1984

Summary. The mutant hairy *(h)* increases the number of sensillae on the *Drosophila* wing. This allows us to quantify a gradient that determines the type of sense organ that forms along the third long vein. Temperature significantly shifts the positional responses to this underlying gradient.

Key words. Positional information; temperature effects; gradients; campaniform sensillae; wing veins; *Drosophila melanogaster.*

Gradients appear to play a central role in determining the placement of structures in a developmental field[2]. *Drosophila melanogaster,* for example, typically has three domed campaniform sensillae on the third wing vein (L3) distal to the crossvein. Although individuals may differ in the relative placement of these sensillae, their average positions are extremely constant.