Transglutaminase and polyamine dependence of effector functions of human immunocompetent cells

The effect of specific inhibitors on lymphocyte proliferation and granulocyte chemiluminescence

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The effects of the transglutaminase inhibitor dansyl cadaverine (DC) and the polyamine antagonist methyl glyoxal-bis-(guanylhydrazone) (MeGbG) on the response of lymphocytes towards allogeneic and lectin stimulation and on the zymosan-induced chemiluminescence of neutrophilic granulocytes was studied. Application of DC resulted in dose-dependent suppression of chemiluminiscence and lymphocyte proliferation; no difference of inhibitory potential occurred with variation of incubation time in the latter system. MeGbG was inactive in granulocytes, but inhibited lymphocyte proliferation; its effect increased with time. The experiments provide further evidence for the importance of transglutaminases and polyamines for the function of immunocompetent cells.

Transglutaminase *Polyamine* *Dansyl cadaverine* *MeGbG* *Lymphocyte* *Granulocyte*

Abbreviations: C, concentration of inhibitors; *I%*, per cent inhibition, calculated as:

$$I\% = 100 - \left(100 \times \frac{\text{activity of inhibited sample} - \text{background}}{\text{activity of uninhibited sample} - \text{background}}\right)$$

ID_{50}, concentration of inhibitor yielding 50% inhibition; LDH, lactate dehydrogenase; MNC, mononuclear leucocytes (lymphocytes and macrophages)

† This paper is dedicated to Professor Gerd Brunner. He postulated that cellular functions (e.g., gene expression) depend on membrane-located information transduction systems activated by external signals, a process for which he coined the term 'membrane impression' [1]. Gerd and his companion, Dr Wolf, both excellent alpinists, did not return from an ascent to the Gasherbrum II peak (8036 m), Northern Pakistan, in early July, 1982

1. INTRODUCTION

Transglutaminases (TGases), comprising a whole family of enzymes with different specificity and functions [2], catalyze the formation of amido bonds, involving the 5-carbamyl moiety of peptidyl glutamine, and an amine (fig. 1). The best-characterized member of this family is blood coagulation factor XIII. TGases are capable of crosslinking proteins on the cell surface [3,4] and in solution [4,5]. TGases are essential for the internalization of specifically bound ligands (receptor-mediated endocytosis) at least in fibroblasts, kidney and tumor cells [6–10].

Evidence has arisen that membrane-associated TGase activity is also related to receptor-mediated functions of immunocompetent cells. It sharply rises after lectin stimulation of mononuclear leuco-

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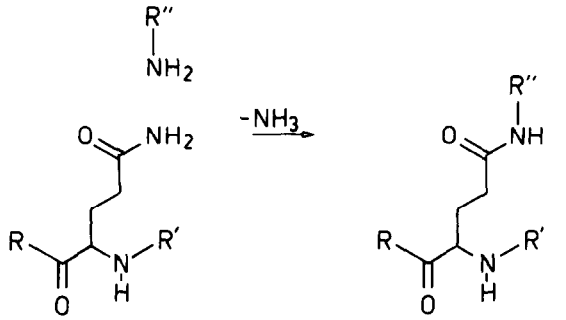

Fig. 1. Schematic representation of TGase-catalyzed reactions: R and R', peptidyl backbone of the glutaminyl susstrate; R'', residue of peptidyl lysine, polyamines or synthetic amine substrates (e.g., dansyl cadaverine).

cyte (MNC) cultures [11], which results in lymphocyte proliferation. TGase activity is also enhanced by immune complex binding to phagocytes [12], which results in an increased oxygen uptake and the production of cytotoxic hydroxyl radicals, singlet oxygen, per- and superoxides [13]. This respiratory burst is induced by appropriate stimulants [14,15]. Whether the relation of TGase activity to these processes is causal or coincidential, i.e., whether immune functions can be suppressed by direct TGase inhibition, has not yet been clarified.

The model compound for TGase inhibition is dansyl cadaverine (DC). It directly competes for the amine binding site [7,8,10] and is covalently incorporated into proteins by TGase action [3,16]. DC inhibits the receptor-mediated endocytosis of insulin, α_2-macroglobulin, polypeptide hormones and virus in various cells [6–8,10].

One of the functions of TGases is the incorporation of physiological polyamines into proteins [2,17]. This post-translational modification can yield both crosslinked and non-crosslinked products [2,17,18]; i.e., polyamines are used for side-chain modifications resulting in unusual amino acids [18] or for the formation of intermolecular bridges [2,17]. Aside from being TGase substrates, polyamines are of vital importance in differentiation, proliferation, and gene expression [19–22]. Thus the polyamine and TGase systems appear to be interlocked, but not identical; interference with polyamine metabolism and inhibition of TGase activity should lead to differing results.

The model compound for polyamine antimetabolites is methyl glyoxal-bis-(guanylhydrazone) (MeGbG). It interferes with synthesis and transport of spermidine [21] and, consequently, spermine. MeGbG has been used as an antileucemic agent not only experimentally, but also clinically [23,24]; it is known to suppress polyamine-dependent [25] lymphocyte proliferation [21,23]. Because of the existence of an intracellular pool of polyamines [26], it should be expected that the maximal inhibitory effect of MeGbG will be reached only after depletion of this pool. Thus far, no kinetic data have been available.

The inhibitory potential of both compounds was tested in the concanavalin A (con-A)-induced as well as allogeneic stimulation of lymphocyte proliferation, measured as incorporation of [^{3}H]thymidine, and in the zymosan-induced respiratory burst of polymorphonuclear leucocytes (PMN), measured as chemiluminescence [27].

2. MATERIALS AND METHODS

2.1. *Separation of cells*

Heparinized blood from healthy donors was centrifuged on an equal volume of sterilized Ficoll for 30 min at 1800 rev./min. The interphase mononuclear leucocytes (MNC) were collected, washed 3 times with RPMI 1640 medium (Boehringer, Ingelheim) and adjusted to 2×10^6 cells/ml. The pellet of the centrifugation, containing erythrocytes and PMN cells, was allowed to settle in a 1% dextran solution for 45 min at $1 \times g$. Pure PMN suspensions were gained from the resulting supernatant by lysis of residual erythrocytes with 0.98% NH_4Cl solution, in order to prevent quenching of chemiluminescence.

2.2. *Procedure of stimulation*

The allogeneic and lectin-induced stimulation experiments of MNC cultures were carried out in microtiter plates (Titertek). Only the central 32 wells of each plate were used, the remaining ones filled with medium.

In con A stimulations, 50 μl cell suspension (10^5 cells) were added to 50 μl con A solution (100 μg/ml, Paesel, Frankfurt), 100 μl 25% heat-inactivated AB serum, and 50 μl inhibitor solutions; 50 μl pure medium was substituted for con A in unstimulated and, for inhibitor solution,

uninhibited controls. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2, using RPMI 1640 medium supplemented with 10^5 U/l penicillin/streptomycin. Medium (50 μl) containing 1 μCi [^{3}H]thymidine was added at 24, 48 or 72 h. After an additional 24 h incubation, cells were harvested on Tritertek fiberglass filters and washed 10 times with 0.9% NaCl. The filters were then dried, placed in small liquid scintillation tubes, covered with 1 ml Insta-fluor liquid scintillation cocktail (Packard) and radioactivity was determined with a Kontron MR-300 counter, using the ^{3}H fix-window with automatic dpm calculation.

In mixed lymphocyte cultures (MLC) peripheral blood cells of two healthy donors were used for responder/stimulator combinations. Responder cell suspension (50 μl, 2×10^6/ml) were added to 50 μl stimulator cells (2×10^6/ml, irradiated with 2500 rad), 50 μl 80% heat-activated AB serum, and 50 μl inhibitor solution (pure medium for uninhibited controls). Autostimulation was excluded by combining responder and stimulator cells of the same donor. [^{3}H]Thymidine was added after 120 h. The further procedure paralleled the con A experiments.

In the chemiluminescence assay, 100 μl PMN cell suspension (5×10^5 cells) were incubated for 10 min in 12 × 47 mm polystyrol cuvettes (Lumac, Düsseldorf) at 37°C in a water bath. Then 100 μl freshly prepared Luminol (Fluka, Buchs) at final conc. 2×10^{-4} M, 50 μl zymosan suspension (final conc. 0.33 mg/ml) or pure medium for unstimulated controls and 50 μl inhibitor solution (pure medium for uninhibited controls) were added. The mixture was kept at 37°C and chemiluminescence was measured in duplicate samples at 15, 30 and 45 min in a Biolumat LB 9500 analyzer (Berthold, Wildbad).

2.3. *Preparation of zymosan suspensions*

Zymosan particles were suspended in physiological saline, heated in a boiling water bath for 20 min, washed twice, resuspended at 2 mg/ml with MEM and kept frozen in small aliquots at −20°C until use.

2.4. *Inhibitors*

DC (Fluka, Buchs) was used at final conc. 10 μM–0.16 mM in both types of MNC cultures, and 0.5 μM – 0.133 mM in the chemiluminescence assay. These ranges had been determined to cover both zero and 100% inhibition in preliminary experiments. Because DC was not readily soluble in medium, it was dissolved in 100 μl/mg 70% ethanol and diluted appropriately with medium. Controls showed that the resulting concentrations of alcohol had no effect in MNC cultures; in the chemiluminescence assay, ethanol at >0.1% reduced photon emission. The inhibition values were corrected accordingly. MeGbG (EGA, Steinheim) was used at 1–60 μM in con A cultures as well as in zymosan experiments, and 62.5 nM–30 μM in MLC.

2.5. *Control experiments*

In the lymphocyte experiments, separate plates were prepared to test the viability of the cells by intra- and extracellular determination of LDH activity, as well as using the eosin dye exclusion test to

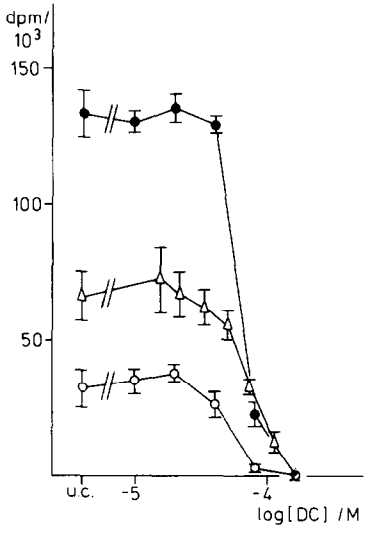

Fig. 2. Dose-dependent inhibition of [^{3}H]thymidine incorporation in lymphocyte cultures by dansyl cadaverine. Bars indicate standard deviation of 4 samples, in uninhibited controls (u.c.) of 8 samples. Statistical significance on the 1% level ($p < 0.01$) required at least 30% inhibition, compared to uninhibited controls. Unstimulated controls accumulated <500 dpm: (○) con A stimulation, 48 h incubation; (●) con A stimulation, 96 h incubation; (△) mixed lymphocyte cultures, 144 h incubation. The 72 h incubation period has not been included.

prove no cytotoxic activity of the inhibitors after 72 h of incubation. Immediately before harvesting, cluster formation of the cultures was evaluated microscopically. Viability of PMN was controlled by dye exclusion.

3. RESULTS

Direct inhibition of TGase activity by DC resulted in dose-dependent suppression of ^{3}H-incorporation in both types of lymphocyte cultures (fig. 2). Inhibition was uniformly characterized by an ID_{50} of 70 μM. In the chemiluminescence assay, dose-dependent inhibition was also obtained; ID_{50}, however, was only 6 μM (fig. 3). DC reduced the viability of lymphocytes slightly, but insignificantly at >40 μM (90% living cells at 160 μM and 94% at 80 μM, compared to 97% in controls), as shown by the eosin dye-exclusion test. The total number of cells did not decrease during incubation with DC even at the highest concentration. LDH in culture medium was not significantly elevated. In the PMN stimulation experiments, viability of cells exceeded 98%.

MeGbG was not active as an inhibitor of chemiluminescence at $\leqslant$60 μM (fig. 3). In the lymphocyte experiments, MeGbG application resulted in dose-dependent suppression of tritium accumulation (fig. 4). In contrast to inhibition by DC, the effect of MeGbG strikingly increased with the duration of incubation: ID_{50} dropped from 15 μM after 48 h to 0.25 μM after 144 h (fig. 5). MeGbG did not diminish total number or viability of cells

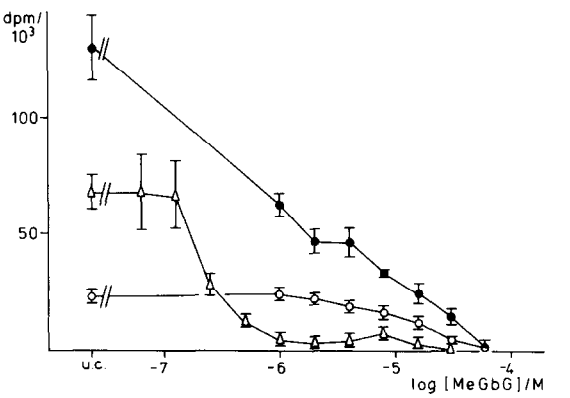

Fig. 4. Dose-dependent inhibition of [^{3}H]thymidine incorporation in lymphocyte cultures by methyl glyoxal-bis-(guanylhydrazone). Conditions and symbols identical to those outlined in fig. 2.

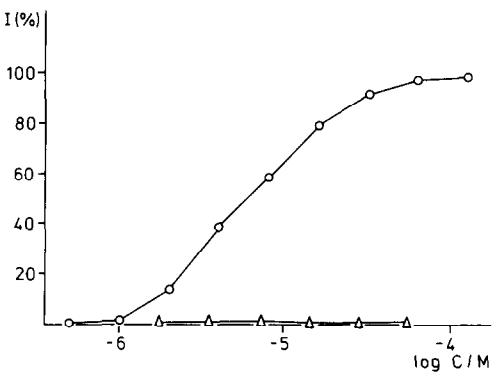

Fig. 3. The effect of dansyl cadaverine in the zymosan-induced chemiluminescence of polymorphonuclear leucocytes (○). Each point represents the mean of duplicate measurements at the maximum of activity (30 min incubation); the values were corrected for background and for the effect of alcohol (section 2). Unstimulated controls yielded 9.5 × 10^3 counts/10 s, uninhibited controls 2.9 × 10^5 counts/10 s. Methyl glyoxal-bis-(guanylhydrazone) (△) was not active as an inhibitor at $\leqslant$60 μM.

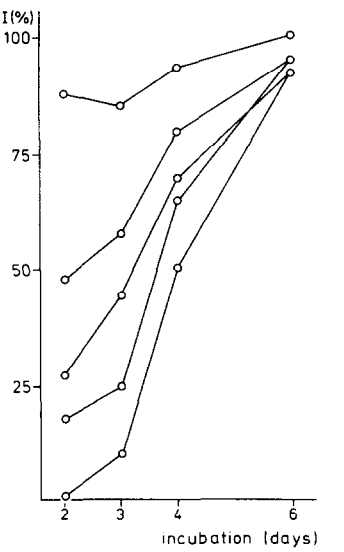

Fig. 5. Time-dependent increase of the inhibitory potential of MeGbG at various concentrations. From top to bottom: 30, 15, 7.5, 3.75 and 1 μM. The values have been compiled from con A (days 2–4) and mixed lymphocyte experiments (day 6).

Table 1

Inhibitory characteristics of the effects of dansyl cadaverine and MeGbG in mononuclear and polymorphonuclear leucocyte cultures

Experiment	Duration (h)	Minimal inhibitory conc. (μM)[a]	ID_{50} (μM)	Total inhibition (μM)[b]
Effects of DC				
Zymosan-induced stimulation of PMN	0.75	2	6	60
Con A stimulation of MNC	48	40	70	160
	72	40	70	160
	96	40	70	160
Mixed lymphocyte culture	144	40	70	160
Effects of MeGbG				
Zymosan-induced stimulation of PMN	0.75	>60	–	–
Con A stimulation of MNC	48	4	15	60
	72	2	10	60
	96	<1	1	60
Mixed lymphocyte culture	144	0.1	0.25	30

[a] Minimal concentration of inhibitor yielding at least 10% inhibition
[b] Lowest concentration of inhibitor totally inhibiting effector function

in the eosin dye-exclusion test, nor did it elevate medium LDH levels in any concentration used.

Cluster formation was inhibited by neither substance in concentrations completely suppressing thymidine incorporation in the lymphocyte cultures. The results of all experiments are summarized in table 1.

4. DISCUSSION

The inhibition of effector functions of immunocompetent cells by DC demonstrates their TGase dependence.

The inhibition of internalization of externally bound macromolecules by DC in fibroblasts [7,8] have led to the postulation [10] of TGase participation in receptor-mediated endocytosis. TGase, activated as a consequence of ligand binding to membrane receptors, was suggested to concentrate and trap ligand–receptor complexes in specialized membrane areas called 'coated pits' as a prerequisite of their internalization as 'receptosomes'. This process characteristically takes place within minutes, and does not necessarily require de novo synthesis of cell structures [28]. The respiratory burst in phagocytes resembles endocytosis in several aspects: it is mediated by receptors, it takes place within minutes, it is TGase-dependent, and it does not necessarily require de novo synthesis of cell structures. Obviously, the specific effect of these similar processes depends on the cell type.

On the other hand, while lymphocyte proliferation is also mediated by membrane receptors and dependent on TGase activity, there are striking differences to fibroblast and granulocyte activation: proliferation requires de novo synthesis of cell structures, and takes place in hours, not minutes.

Apparently, TGases fulfill the requirements of an information transduction system: a uniform initiation step (receptor-mediated enzyme activation) is able to produce entirely different effects in different cells. The existence and the universal importance of such transduction systems was postulated by Gerd Brunner:

> '... *the cell membrane, by transduction of signals from the outside, acts as a central regulatory point and posesses an equivalent counterpart function to the genome*' [1]

The comparison of IC_{50} values of DC inhibition of these processes (300 μM in fibroblasts [8], 70 μM in mononuclear cells and 6 μM in granulocytes) indicates that specificity is achieved by substrate-

specific susceptibility, most plausibly explained by the existence of isoenzymes.

The time-dependence of MeGbG inhibition in the lymphocyte experiments and its inactivity in the PMN test demonstrate the incongruency of TGase and polyamine systems with respect to receptor-mediated activation. The kinetics indicate that turnover and depletion of the pre-existing pool of polyamines proceed slowly. Half-lives of 12–42 days were reported [26], but are not comparable as turnover in organs, not in cell culture, were studied; we were not able to sustain lymphocyte cultures long enough to reach a plateau in the time-course of MeGbG inhibition, indicating that even after 6 days, the polyamine pool is not totally depleted. The consequences of these results have been anticipated by the empirical finding that weekly, rather than daily, application of an overall dose reduced by 30% enhances the therapeutic index of the drug [24].

We cannot yet decide whether phagocyte chemiluminescence involves the utilization of polyamines. This process may be completely independent of the polyamine system, or the depletion of the pool may be negligible, due to the short duration of the respiratory burst. However, even if chemiluminescence is not independent of the presence of polyamines, it is certainly independent of their synthesis and transport. This circumstance, which disentangles the connection of TGase and polyamine systems, the rapidity and simplicity of procedure, and the quantitative nature of the results, make phagocyte chemiluminescence a suitable model for the functional study of TGase-dependent processes.

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