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NYLON WOOL COLUMN – A TOOL FOR OBTAINING MONOKINE-RICH ELUATES IN THE ABSENCE OF SERUM

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Diverse conditions for stimulating human mononuclear cells to release thymocyte costimulatory factors were tested for their contribution to the generation of supernatants containing high titers of these monokines. Activity titers increased with LPS concentration, reaching a plateau between 1 and 10 μg/ml. Indomethacin did not modify the monokine release, but the assay for thymocyte costimulatory activity was substantially affected by inhibitory substances produced by the monocytes in the absence of indomethacin. The use of nylon wool columns to trap the cells was shown to be effective in raising cellular densities without decreasing activity titers. As a result, the yield per cell could be maintained even in the absence of serum, an important step toward the goal of purifying bioactive peptides from crude broths.

Key words: nylon wool columns – human monokine production – thymocyte costimulatory assay – LPS – peripheral blood monocytes

Conditioned media from stimulated monocytes and macrophages have been used as a source of polypeptides involved in the regulation of immunological and inflammatory reactions. From these media were first identified and purified, for instance, interleukin 1 (IL-1) (Gery & Waksman, 1972), tumour necrosis factor (TNF) (Chen et al., 1985), colony stimulating factors (CSFs) (Chervernick & Lo Buglio, 1972; Messner et al., 1973) and interleukin 6 (IL-6) (Van Damme et al., 1985). More recently, novel factors present in lipopolysaccharide (LPS)-stimulated monocyte supernatants have been reported to have chemotactic and stimulatory effects for neutrophils (Yoshimura et al., 1987; Davatelis et al., 1988; Peveri et al., 1988; Van Damme et al., 1988), suggesting that crude monokine-containing broths may still have potential as a source of biologically active molecules, including monokines that have not yet been characterized.

With the goal of obtaining human monocyte supernatants with high monokine activity, we have varied a number of parameters such as LPS dose, cell density, medium, time of harvesting and presence or absence of indomethacin. The murine thymocyte costimulatory assay, which is capable of detecting at least human IL-1 and IL-6 activities (Helle et al., 1988; Lotz et al., 1988), was used to monitor monokine release.

MATERIALS AND METHODS

Animals – Thymus donors were male C3H/HeJ and Balb/c mice 6 to 8 weeks old, bred in the animal-care facility of the Basic Research Center.

Reagents – All reagents were purchased from Sigma Chem. Co., (St. Louis, MO, USA) unless stated. Lipopolysaccharide was a trichloroacetic extract of the Escherichia coli 0111.B4 strain. Stock solutions contained 4 mg/ml in culture-grade (Milli-Q) water (Millipore, São Paulo, Brasil) and were sterilized in an autoclave (115°, 30 min). Fetal calf serum (FCS) was obtained from Gibco Laboratories (Grand Island, NY, USA). A pool of human A^+ serum (HA^+S) was obtained from the National Cancer Institute Blood Bank. Both sera were inactivated at 56 °C for 40 min. RPMI 1640 medium and Dulbecco's modified Eagle's medium (DMEM) were supplemented respectively with 2 and 4 mM of freshly added glutamine, plus penicillin-G (100 U/ml), streptomycin sulphate (100 μg/ml) and 5×10^{-5} M 2-mercaptoethanol. In some experiments, N-2-hydroxyethyl-piperazine N'-2-ethanesulphonic acid buffer (HEPES)

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was added from a 1 M stock solution, pH 7.3. Indomethacin (INDO, 5 mg/ml) was freshly prepared in analytical grade ethanol and diluted for use in medium containing 5% (v/v) serum.

Peripheral blood mononuclear cells – Human peripheral blood mononuclear cells (PBMN) were isolated from buffy coats of healthy A^+ blood donors by centrifugation (400 xg, 30 min, 18 °C) on Histopaque d = 1.077 (Boyum, 1968). PBMN cells were washed four times (500 xg, 7 min) with Eagle's basal medium (washing medium) and re-suspended in RPMI 1640 or DMEM containing 5% FCS.

Induction of monokines – PBMN cells (5 x 10^6 cells in 1 ml RPMI 1640 plus 5% FCS) were plated on 2 cm^2, 24 macrowell plastic plates (Falcon, Becton & Dikinson, CA, USA), and left for 2 h at 37 °C in order to allow monocyte attachment. Loose cells were withdrawn by aspiration and the adherent monolayers were gently washed with prewarmed (37 °C) RPMI 1640 plus 5% FCS, to remove the remaining non-adherent cells. Monolayers were stimulated in different ways in RPMI 1640 or DMEM at 37 °C for 24-72 h in a humidified atmosphere of 6% CO_2 in air. Supernatant samples were withdrawn aseptically and kept frozen (–20 °C) until testing for thymocyte costimulatory activity (TCAc). This procedure was modified in some experiments (Table III), in order to achieve higher cell densities, as follows: 40 ml aliquots of a PBMN cell suspension containing 5 x 10^6 cells/ml in RPMI 1640 plus 5% serum were incubated in γ-irradiated, flat-bottomed plastic bird baths (80 cm^2, 4 cm deep, functioning as deep petri dishes), for 2 h at 37 °C to allow monocyte attachment. Loose cells were removed and the monocyte monolayers were washed with prewarmed (37 °C) washing medium. Cells were then detached in cold medium by the use of a rubber policeman, centrifuged (500 xg, 7 min), re-suspended in DMEM and distributed in 24 well plates before being stimulated in different ways for monokine production. Supernatant samples were collected and stored as described above.

Monokine induction in nylon wool columns – Nylon wool (Fenwal Lab., Illinois, USA) was extensively washed in 0.1N HCl and culture-grade water. Dried wool (0.3 g) was packed into 3 ml controlled-flow glass syringes, autoclaved and equilibrated with DMEM plus 5% FCS. Two ml of PBMN cells suspended in the same medium were layered onto each column at 0.5 ml/min. Syringes were plugged and incubated for 2 h at 37 °C, to allow monocytes to adhere. Then the unattached cells were washed out with 15 ml of pre-warmed medium (37 °C), the cells attached to the nylon wool were slowly perfused with the definitive stimulating medium containing LPS and the additions specified for each experiment, and the syringes were plugged for a 24 h incubation at 37 °C. Conditioned media were pressed out of the columns, centrifuged at 1,000 xg for 10 min to remove cells and for some experiments were dialysed in 3.5 KDa cutoff dialysis bags (Spectrum Med. Ind., Gardena, CA, USA) against 3 changes of 10 mM phosphate buffered saline, pH 7.3, plus 1 change of 20 mM HEPES buffered DMEM. After dialysis, the conditioned media were sterilized by filtering through GV membranes (Millipore, São Paulo, Brasil), and kept at –20 °C until assayed for thymocyte costimmulatory activity.

Thymocyte costimulatory activity (TCAc) – Conditioned media were serially diluted 1:3 (one volume in two of fresh medium) eight to 12 times, in duplicate, with RPMI 1640 medium in 96-well, flat-bottomed plates (Petécil, São Paulo, Brasil). Phytohemagglutinin-M (PHA), FCS and thymocytes were added to final concentrations of 20 μg/ml, 5% and 7.5 x 10^6/ml, respectively, in a final volume of 0.2 ml/well. The plates were incubated at 37 ° in a humidified atmosphere of 6% CO_2 in air for 48 h, and then for 24 h after adding 0.5 μCi of ^{3}H thymidine (^{3}H TdR, sp. act. 2 Ci/mmole, Dupont, Boston, MA). Cell suspensions were harvested onto electrophoresis filter paper, dried, washed three times in tap water and dried again. The ^{3}H TdR incorporated into DNA was measured in a liquid scintillation counter (Beckman LS-100) and expressed in terms of mitogenic units (dilution of the conditioned medium that yields double the number of counts in the control containing fresh medium, PHA, FCS and thymocytes) (Svenson & Bendtzen, 1988). Experimental errors were evaluated from a complete dilution curve of each conditioned medium, using the SD of each experimental point to trace parallel curves ± SD above and below the data points. Extrapolated to the abscissa, these parallel curves furnish the titer limits at the level of the dilution giving double of the control counts. This method of evaluating titers has

the disadvantage of giving larger errors in mitogenic units at higher dilutions (e. g. when assaying high-titered conditioned media) for the same error in cpm. Nevertheless, it allows comparison among conditioned media that are inhibited to different extents, or for other reasons differ in maximal activity. Titers can only be compared within the same titration experiment and among supernatants from the same blood donor. The 2 strains of mice used in our experiments differ in responsiveness of their thymocytes to LPS. However, under the conditions of our TCAc assay, addition of LPS up to 100 μg/ml did not alter proliferation in PHA controls (Dalmau & Freitas, 1990).

RESULTS

Thymocyte costimulatory activity from monocyte monolayer supernatants – It has been reported that prostaglandins (PGs) impair both IL-2 (Chouaib & Fradelizi, 1982; Tilden & Balch, 1982; Walker et al., 1983) and IL-1 production (Herman & Rabson, 1984) by human mononuclear cells, as well as murine thymocyte proliferation *in vitro* (Hayari et al., 1985). For this reason we tested the effect of including a PG synthesis inhibitor in the monocyte cultures during stimulation. Figure 1 shows that thymocyte proliferation was inhibited for the less diluted samples of conditioned medium when supernatants were generated in the absence of indomethacin. This result could mean that TCAc release was impaired, or that stimulation of thymocyte proliferation was counteracted by the presence of inhibitors (e. g., PGs) in these conditioned media. In later experiments, results obtained with nylon wool column eluates generated in the presence and absence of INDO (Fig. 3) supported the second of these two hypotheses.

When monocytes were stimulated with increasing doses of LPS, TCAc release reached a plateau with 1 μg/ml LPS in the absence (Experiment 1) or between 1 and 10 μg/ml in the presence (Experiment 2) of 1 μg/ml indomethacin (Table I). Table II shows that TCAc increased three-fold with the addition of 5% HA^+S or 10% FCS.

TCAc was assayed in supernatants from monocyte monolayers obtained under diverse conditions of culture medium (RPMI 1640 or DMEM), time of harvesting (24, 48 or 72 h) and additional buffer (0 or 20 mM HEPES). In

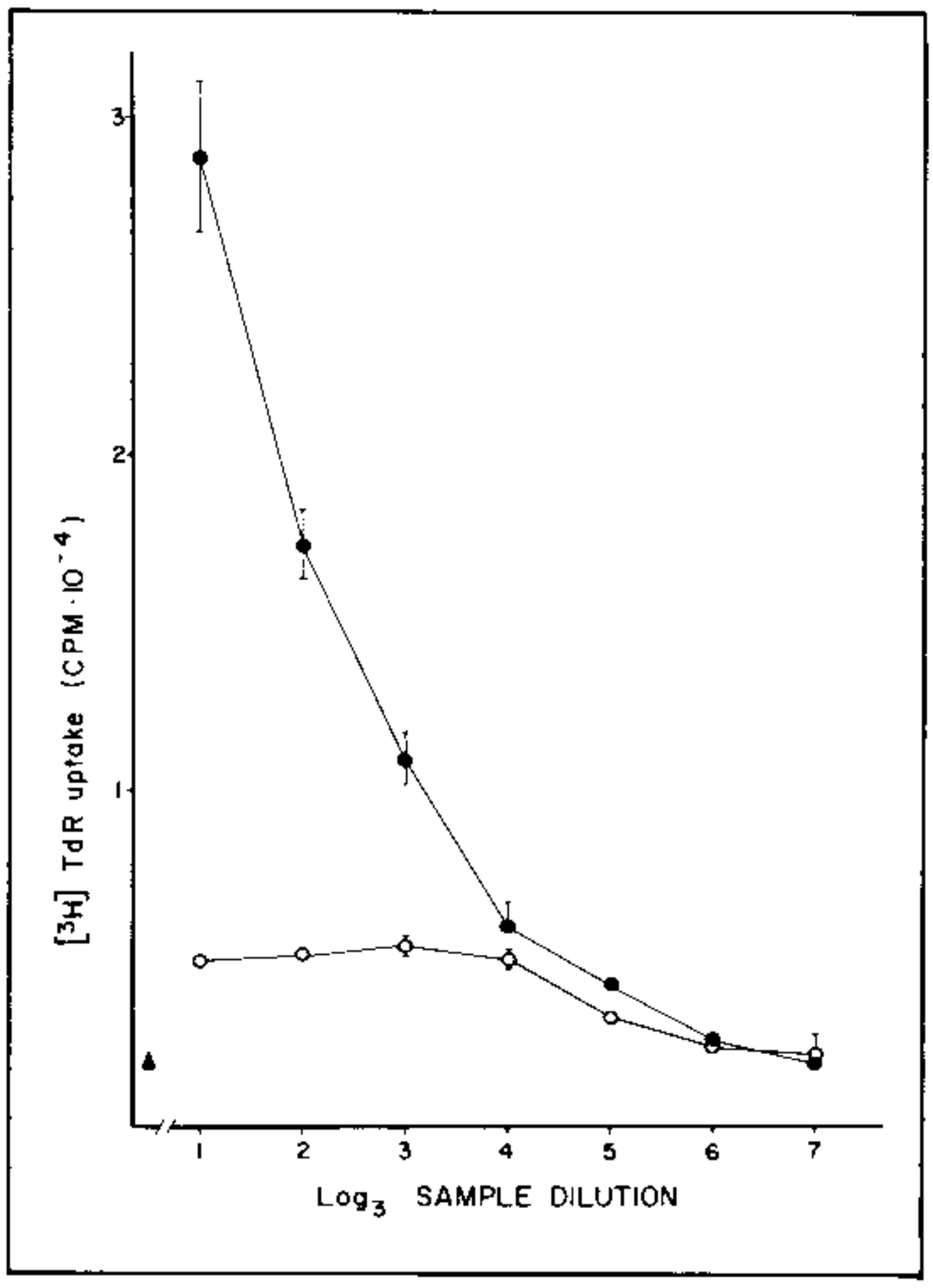

Fig. 1: effect of indomethacin addition to LPS-stimulated monocytes during generation of monokine-containing supernatants. Monocyte monolayers were stimulated with 10 μg/ml LPS in 1 ml of DMEM plus 5% HA^+S for 48 h at 37 °C, in the presence (–●–) or absence (–o–) of 1 μg/ml indomethacin. Supernatants were assayed for their costimulatory activity using Balb/c thymocytes. Control for the mitogenic assay, (▲) 20 μg/ml PHA alone. Results expressed in cpm (mean ± SD).

TABLE I

Thymocyte costimulatory activity titers obtained in response to different LPS doses

LPS concentration (μg/ml)	TCAc titers (units/ml)	
	Exp. 1	Exp. 2
–	19 (18-22)	⩽3
0.01	ND	24 (22-26)
0.10	ND	133 (126-140)
1.00	175 (129-206)	260 (218-303)
10.00	175 (157-195)	338 (329-357)
100.00	179 (148-227)	320 (280-377)

Monocyte monolayers were stimulated with different LPS doses for 48 h at 37 °C in 1 ml of DMEM containing either 5% FCS in the absence of indomethacin (Exp. 1), or 2.5% HA^+S in the presence of 1 μg/ml indomethacin (Exp. 2). TCAc assay with Balb/c thymocytes. The titer limits (in parentheses) were estimated from ± 1 SD of the thymidine incorporation curve as described in Materials and Methods. ND, not determined.

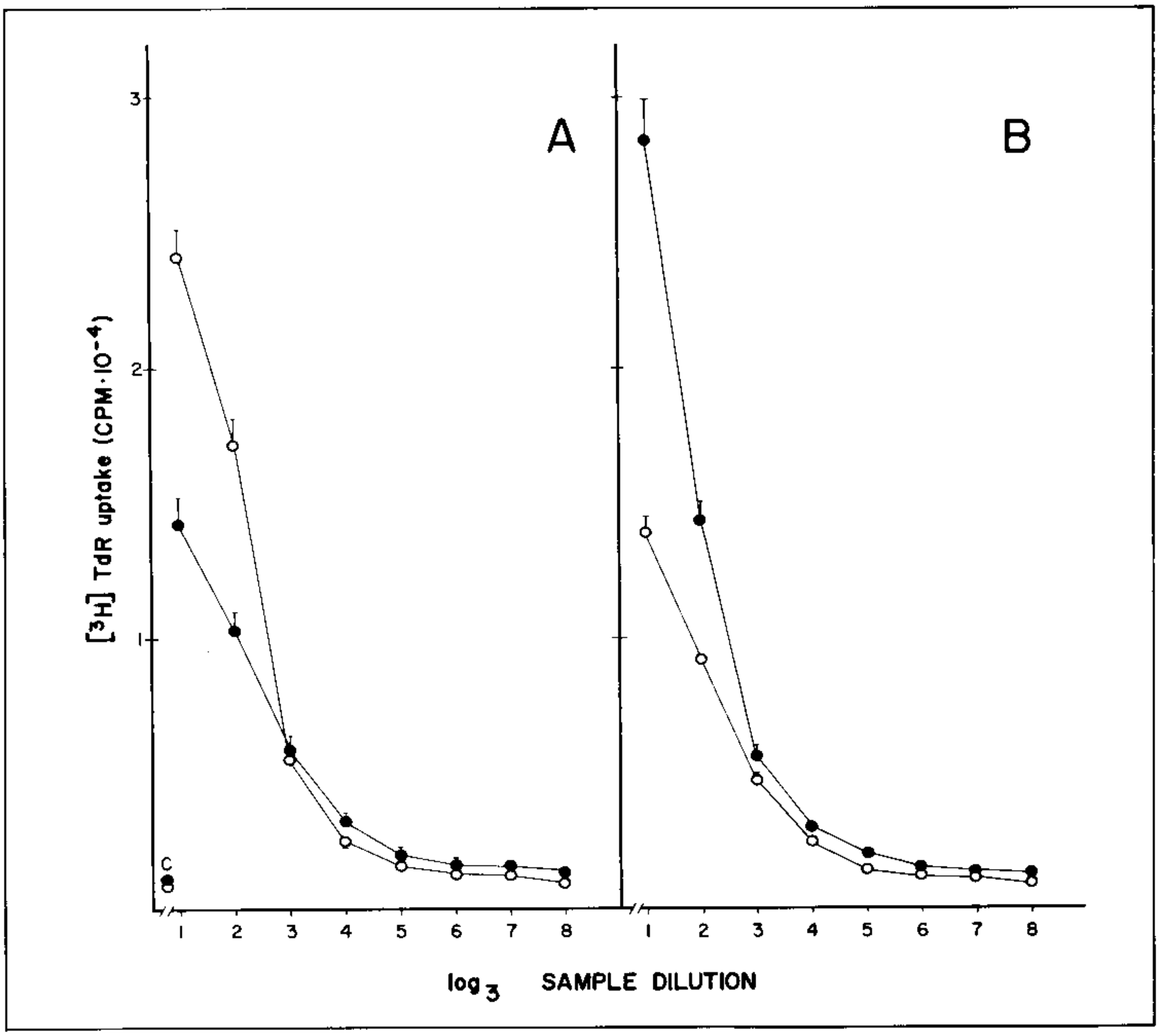

Fig. 2: influence of medium on production and measurement of thymocyte costimulatory activity. Monocyte monolayers were incubated either in RPMI 1640 (Fig. 2A) or in DMEM (Fig. 2B) for 24 h at 37 °C with 10 μg/ml LPS plus 1 μg/ml indomethacin and 5% FCS. Balb/c thymocytes were used in the costimulatory assay, that was carried out either in RPMI 1640 (–●–) or in DMEM (–o–). C, controls. Results expressed in cpm (mean ± SD).

these experiments, the concentrations of LPS (10 μg/ml), HA^+S (5%) and indomethacin (1 μg/ml) were kept constant. Similar titers (between 3^4 and 3^5 mitogen units/ml) were obtained for supernatants from cultures under all of these conditions.

While the choice of medium did not seem to be important for the generation and recovery of TCAc from supernatants, it was important for the TCAc assay itself. Figure 2 shows the titration curves obtained with conditioned media produced in RPMI 1640 (Fig. 2A) or in DMEM (Fig. 2B), when assayed for their TCAc titers either in RPMI 1640 or in DMEM.

TABLE II

Influence of serum on thymocyte costimulatory activity from monocyte monolayers

Serum added (% v/v)	TCAc titer (units/ml)
–	113 (101-129)
2.5% HA-S	230 (220-236)
5.0% HA-S	338 (329-357)
10.0% HA-S	409 (329-483)
10.0% FCS	347 (295-398)

Monocyte monolayers derived from 5 x 10^6 PBMN/ml were stimulated with 10 μg/ml LPS in DMEM containing 1 μg/ml indomethacin and different amounts of HA^+S or FCS, for 48 h at 37 °C. TCAc assay with Balb/c thymocytes. The titer limits (in parentheses) were estimated as described in Material and Methods.

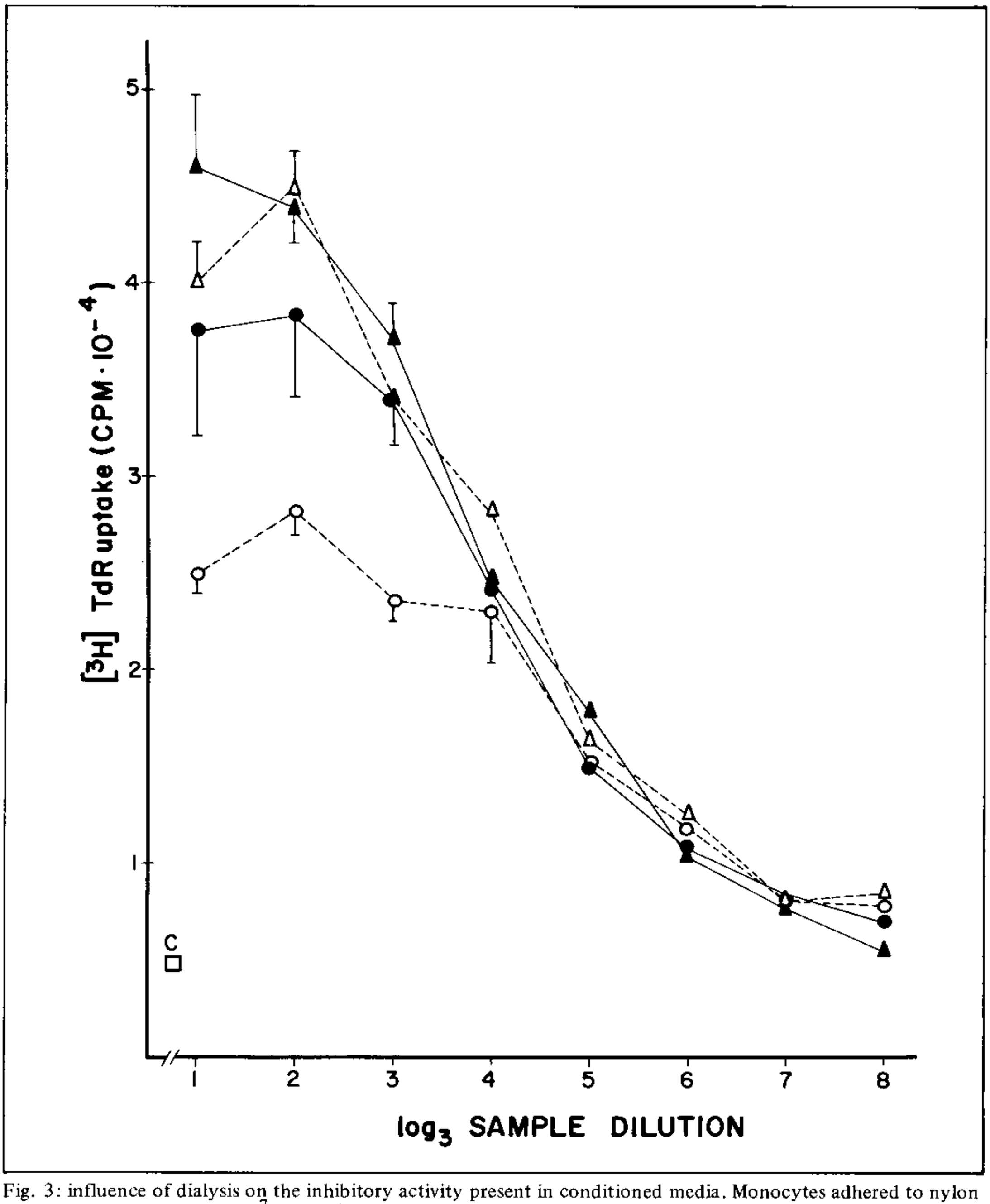

Fig. 3: influence of dialysis on the inhibitory activity present in conditioned media. Monocytes adhered to nylon wool columns (from 5 x 10^7 PBMN/ml, 2 ml per column) were stimulated with 5 μg/ml LPS in DMEM plus 5% FCS in the presence (△, ▲) or absence (o , ●) of 1 μg/ml indomethacin. Curves correspond to conditions 1 to 4 of Table VI. Part of each conditioned medium was dialysed (closed symbols) and part was not (open symbols). The comitogenic activity was measured using C3H/HeJ thymocytes. Control for the comitogenic assay (□), 20 μg/ml PHA alone. Results expressed in cpm (mean ± SD).

Attempts were made to increase the TCAc titers by raising the density of cells under stimulation. Monocytes were detached from monolayers, counted for viability under phase contrast microscopy, and seeded in 2 cm^2, 24-well plates at different cell densities, before being stimulated with 10 μg/ml LPS in DMEM containing 5% HA^+S and 1 μg/ml indomethacin. The yield per cell decreased when the density exceeded 5 x 10^6 cells/ml in each well

(Table III). Yield per cell improved only slightly when the medium contained extra buffer (20 mM HEPES) or when the volume of nutrient medium was increased to 2 ml per well; the absence of serum substantially decreased the yield (Table III).

TABLE III

Thymocyte costimulatory activity in supernatants from detached monocytes

Monocytes (no./well)	HEPES (20 mM)	TCAc titer (units/ml)	Yield (units/10^6 monocytes)
2.5×10^{5}[5]	–	83 (82- 84)	332
5.0×10^{5}	–	230 (152-273)	460
1.0×10^{6}	–	236 (206-271)	236
2.0×10^{6}	–	236 (232-240)	118
4.0×10^{6}	–	483 (329-555)	121
2.0×10^{6}	+	470 (320-635)	235
4.0×10^{6}	+	618 (554-635)	155
2.0×10^{6}[a]	–	136 (126-152)	68
2.0×10^{6}[b]	–	338 (328-348)	169

Different numbers of detached monocytes were stimulated for 48 h with 10 μg/ml LPS in 1 ml DMEM plus 5% $HA^{+}S$ and 1 μg/ml indomethacin, in diverse conditions, as indicated. TCAc assay with Balb/c thymocytes. Titer limits (in parentheses) were estimated as described in Materials and Methods.

[a] Without serum
[b] 2 ml of medium

Thymocyte costimulatory activity in nylon wool column eluates – In order to increase monocyte densities without subjecting the cells to the injurious procedure of detachment, PBMN were presented with larger attachment surfaces by using nylon wool columns (NWC).

Table IV shows the TCAc titers obtained when increasing numbers of PBMN were applied to NWC. TCAc titers increased in proportion to cell density, so that the yield per monocyte was constant up to the highest density tested (5×10^{7} PBMN/ml). As stated in Materials and Methods, valid comparisons among conditioned media obtained under different conditions were limited to data collected from the same titration experiment and supernatants from the same blood donor, due to inter-assay and inter-donor variations described elsewhere (Lepe-Zuniga & Gery, 1984). For this reason, we compared supernatants obtained by LPS stimulation of monolayers with those obtained from NWC using monocytes from the same blood donor. Although a tenfold higher cell density was used in the NWC system, titers of the NWC eluates were also higher, so that the yield per cell exceeded (Experiment 2) or at least matched (Experiment 1) that obtained from the monolayers (Table V).

TABLE IV

Thymocyte costimulatory activity in eluates from nylon wool columns

No of PBMN applied per ml of medium	TCAc titer (units/ml)	Yield[a] (units/10^6 monocytes)
6.25×10^{6}	160 (136- 192)	170
1.25×10^{7}	312 (268- 348)	166
2.50×10^{7}	656 (536- 784)	175
5.00×10^{7}	1192 (1168-1220)	159

Mononuclear cells were applied to NWC to allow monocyte attachment according to Material and Methods. Adhered cells were stimulated with 40 μg/ml LPS in DMEM plus 5% FCS for 24 h at 37 °C. Media were then eluted from columns, freed from cells, dialysed and kept frozen until being assayed for comitogenic activity using Balb/c thymocytes. Titer limits (in parentheses) were estimated as described in Materials and Methods.

[a] Assuming that monocytes represent 15% of total PBMN applied.

Attempts were made to optimize TCAc yields from NWC by varying LPS doses in the presence or absence of indomethacin and serum. Aliquots of some eluates were dialysed as described in Methods with a view to eliminating putative low-molecular-weight inhibitors of the TCAc assays. Table VI shows that a 16-fold increase in LPS concentration did not modify TCAc recovery significantly. Moreover, in contrast to the results obtained with monolayers and detached monocytes, the absence of serum did not cause a decrease in titer. The high titers shown in this table probably reflect the greater sensitivity of the C3H/HeJ thymocytes used in this assay, compared with the Balb/c thymocytes used in Tables I to V.

Figure 3 shows that dialysis of eluates produced in the absence of indomethacin virtually eliminated the inhibition of thymocyte proliferation that was observed at low dilutions of the eluates. Conditioned media produced in the presence of indomethacin, on the other hand, were unaffected by dialysis.

TABLE V

Thymocyte costimulatory activity in supernatants from monocyte monolayers as compared to NWC eluates

Exp.	TCAc titers in monolayer supernatants (units/ml)	Yield[a] (units/10^6 monocytes)	TCAc titers in NWC eluates (units/ml)	Yield[a] (units/10^6 monocytes)
1	140 (125-160)	186	1192 (1168-1220)	159
2	179 (175-183)	238	3116 (2792-3632)	415

Monocyte monolayers were derived from 5 x 10^6 PBMN/ml and monocytes from the same blood donor were derived from NWC to which 2 ml of PBMN (5 x 10^7 cells/ml) were applied. Cells were stimulated with 40 μg/ml LPS in DMEM plus 5% FCS (Exp. 1) or with 10 μg/ml LPS in DMEM plus 1 μg/ml indomethacin and 5% HA + S (Exp. 2) for 24 h at 37 °C, and TCAc assays were carried out with Balb/c thymocytes. Titer limits (in parentheses) were estimated as described in Materials and Methods.

[a] Assuming that monocytes represent 15% of total PBMN applied.

TABLE VI

Thymocyte costimulatory activity in eluates from nylon wool columns

Condition of incubation	LPS (μg/ml)	INDO (1 μg/ml)	Dialysis	TCAc titers (units/ml)
1	5	–	+	6872 (5516-7748)
2	5	–	–	6576 (6292-7024)
3	5	+	+	4876 (4728-5892)
4	5	+	–	6872 (6576-7188)
5	20	–	+	5764 (5700-5892)
6[a]	20	–	+	8372 (8192-8560)
7[b]	20	–	+	7840 (5516-9992)
8	80	–	+	5164 (3632-6872)
9	80	–	–	7024 (6156-8012)
10	80	+	+	5012 (4524-5636)
11	80	+	–	7840 (6432-9764)

Monocytes adhered to nylon wool columns were derived from 5 x 10^7 PBMN/ml (2 ml per column). Cells were stimulated under various conditions in DMEM plus 5% FCS for 24 h at 37 °C. Eluates from columns were dialysed or not, as shown, and tested for costimulatory activity with C3H/HeJ thymocytes. Titer limits (in parentheses) were estimated as described in Materials and Methods.

[a] With 20 mM HEPES.
[b] Without serum.

DISCUSSION

The different conditions for generating and recovering monokines described in this report were evaluated using the thymocyte costimulatory assay.

This assay has been shown to detect IL-1 (Gery et al., 1981), IL-2 (Shaw et al., 1978), IL-6 (Helle et al., 1988; Lotz et al., 1988), murine but not human TNF (Ranges et al., 1988), and the recently reported cytokine IL-7 (Okazaki et al., 1989). Murine TNF has a synergistic effect with IL-1, IL-2 and IL-4 in this assay (Ranges et al., 1988). Synergism was also observed between IL-4 and IL-2 (but not between IL-4 and gamma interferon, granulocyte monocyte colony stimulating factor, IL-3 or IL-1) (Carding & Bottomly, 1988). In our experiments, it is not likely that either IL-2 or IL-4 would be present as contaminants in the monocyte monolayer supernatants. However, they could be present in the NWC eluates, since this kind of matrix is known to trap many B and some T lymphocytes in addition to monocytes. However, we were not able to detect

amounts of IL-2 or IL-4 activity by the cytotoxic T lymphocyte L (CTLL) proliferation assay (Grabstein et al., 1986), in either monolayer or NWC conditioned media, even when produced in the presence of indomethacin. Therefore, the TCAc we detect in human monocyte supernatants can tentatively be ascribed to the cytokines IL-1 or IL-6, or both (Elias et al., 1989). IL-7 has not yet been found in monocyte supernatants.

Nylon wool columns proved to be effective in raising cellular densities without cellular damage. Compared to monolayers, this system has the advantage of producing media with higher titer (Table V) and specific activity, since conditioned media of comparable titer can be generated in the absence of serum (Table VI). The data of Tables IV and V show that up to 5×10^7 PBMN per ml could be applied without a decrease in yield per cell. Since a limit was not reached in these experiments, it may be feasible to use cell densities even higher than those we tested.

Some groups use to compare IL-1 levels in monocyte or macrophage supernatants by assaying them for thymocyte comitogenic activity in cpm at a single concentration. Otherwise, a complete dilution curve is generated in order to define the concentration of the supernatant for half-maximal activity. It is clear from Figs 1-3 that both procedures can lead to erroneous estimates of the TCAc titers, since proliferation is affected by inhibitory factors such as PGs, by dialysis, and even by the medium in which the monocytes were stimulated. This source of error may account for some of the contradictory findings in the literature, such as those related to the participation of PGs in IL-1 production. The present results (Fig. 3 and Table VI) showed that similar levels of TCAc could be recovered from human monocytes stimulated with LPS either in the presence or absence of indomethacin. These observations are in agreement with those of Hayari et al. (1985), who used murine peritoneal macrophages, and by Khansari et al. (1985) who used human monocytes. On the other hand, Kunkel et al. (1986) used conditioned media from LPS-stimulated, resident murine peritoneal macrophages or blood-derived monocytes and showed that the levels of IL-1 (assayed as TCAc) and MO-derived fibroblast growth factor were enhanced by cyclooxygenase inhibitors and reduced by exogenous PGs, even after dialysis. In the present study, in accordance with Hayari et al. (1985), dialysis abolished the inhibitory activity that characterizes conditioned media generated in the absence of INDO. The experiments of Fig. 3 and Table III show that, among the low-molecular-weight substances present in monocyte supernatant, the most important inhibitor(s) of thymocyte proliferation may be products of the cyclooxygenase pathway, which can be removed by passage through and/or adsorption to dialysis bags.

Conditioned media generated on NWC in the absence of indomethacin yielded less inhibited titration curves than media generated from monocyte monolayers without INDO (data not shown). It may be that since B cells as well as monocytes adhere to NWC they may absorb PGs from the media (Chouaib & Fradelizi, 1982).

In respect to the evaluation of inhibition of the TCAc assays, we would like to emphasize the importance of using low specific activity ^{3}H TdR when measuring TCAc in monocyte and macrophage conditioned media, in order to minimize competition by non-radioactive thymidine present in the supernatants (Stadecker, 1981) or arising from dead thymocytes in the TCAc assay. Svenson & Bendtzen (1988) showed that an "inhibitor" of IL-1 in the TCAc assay could be attributed at least in part to a decrease in specific activity of ^{3}H TdR as unlabelled thymidine was released by dead thymocytes.

In this study only parameters that might affect the recovery of extracellular TCAc were considered. Further investigation of parameters relevant to the overall recovery (intra + extracellular), based on the association of the LPS stimulus to other stimulants may lead to additional improvements (Gery et al., 1981; Lepe-Zuniga & Gery, 1984).

Starting from a crude monocyte supernatant, it should be possible to screen for factors others than those detected by the TCAc assay, provided that suitable assays are used. For this purpose, stimulation of adherent cells on NWC appears to be a superior technique for obtaining high-titered broths in the absence of serum, thus facilitating fractionation and characterization of new factors.

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