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A MODIFIED ASSAY FOR MEASURING THYMOCYTE CO-STIMULATORY ACTIVITY

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The observation that murine thymocytes increase their proliferation to interleukin 1 (IL-1) in the presence of phytohemagglutinin (PHA) when pre-incubated with interleukin 2 (IL-2) allowed the introduction of a modified assay for the measurement of IL-1 or the search of thymocyte-inducing proliferative activities in biological samples. Pre-incubation of thymocytes for 24 hr with 50 u/ml IL-2, followed by washings, elicited their maximal response to IL-1 in the usual lymphocyte activating factor (LAF) assay. This suggests that sequential events lead to thymocyte activation. The responsiveness is three to five fold greater than, and the total time of assay is the same as that of the LAF assay. Interestingly, pre-incubation with IL-2 renders thymocytes more sensitive than responsive to crude monocyte conditioned media. The use of the MTT colorimetric method for the assessment of thymocyte proliferation, and of the lectin jacalin as a co-mitogen are suggested as alternatives to be used in co-stimulatory assays.

Key words: thymocyte – co-stimulatory assays – interleukin 1 – interleukin 2 – MTT – Jacalin

The lymphocyte activating factor (LAF) assay was initially introduced to measure interleukin 1 (IL-1) activity in biological samples (Gery et al., 1972). It exploits the ability of IL-1 in co-inducing, with an appropriate amount of lectin, the proliferation of murine thymocytes at high densities (2.5-7.5 x 10^6 cells/ml). A modification of this assay was later proposed, for determining interleukin 2 activity (Shaw et al., 1978). Such "thymocyte co-stimulatory assay" employed a ten fold lower number of thymocytes than the LAF assay, a condition where IL-1 is not active. Both co-mitogenic assays are now known to be positively affected by other cytokines such as murine tumor necrosis factor alpha (TNF-α) (Ranges et al., 1988), interleukin 4 (IL-4) (Conlon et al., 1989), interleukin 6 (IL-6) (Helle et al., 1988), and interleukin 7 (Conlon et al., 1989; Okasaki et al., 1989). Synergism of TNF-α with IL-1, IL-2 or IL-4 (Ranges et al., 1988), IL-2 with IL-4 (Carding & Bottomly, 1988), and IL-1 with IL-6 (Elias et al., 1989) has been also reported in the LAF assay. It was absent in combinations of IL-4 with interferon gamma, granulocyte-monocyte colony stimulating factor, interleukin 3, or IL-1 (Carding & Bottomly, 1988).

The mitogen-dependent proliferative response of bulk thymocyte suspensions to exogenous IL-1 has been essentially ascribed to mature (ready to leave the thymus) medullary peanut agglutinin-negative lymphocytes. It would correspond both to this subpopulation capabilities of producing IL-2 in response to lectins (Conlon et al., 1982; Hanson et al., 1983) and of enhancing IL-2 receptor (IL-2R) expression under the synergistic action of IL-1 and IL-2, in a lectin-independent way (Mannel et al., 1985; Falk et al., 1989). The later observation prompted Falk et al. (1987) to devise a co-stimulatory assay, in the absence of lectins, in which IL-2R-inducing capability of IL-1 is the only activity titered, in the presence of a saturating amount of IL-2. This assay has the advantage of allowing the measurement of IL-1 activity in samples containing IL-2. However, it remains the disadvantage of being putatively affected by other cytokines such as IL-6 or TNF-α, that also increase bulk thymocyte proliferation in saturating amounts of IL-2, in the absence of lectins (Suda et al., 1990). Actually, TNF-α is also known to induce IL-2R expression on thymocytes (Lowenthal et al., 1989).

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Although lacking in specificity, murine thymocyte comitogenic assays remain as simple and reliable systems for the initial screening of the above mentioned or even other yet unknown factors in biological fluids and culture supernatants. Using the thymocyte costimulatory assay, for example, Elias et al. (1989) demonstrated the modulation of IL-6 secretion by IL-1 in cultured fibroblasts, while we and others observed a spontaneous IL-1 release by peripheral blood mononuclear adherent cells derived from rheumatoid arthritis patients (Sabino et al., 1989; Goto et al., 1990).

In the present work we show that pre-incubation of thymocytes with IL-2 increases both their responsiveness to IL-1 and their sensitivity to human monocyte conditioned media in the classical LAF assay. These observations, while raising additional questions concerning the relationship among cytokines and thymocyte subpopulations, provide an alternative assay for the measurement of IL-1 or other thymocyte proliferative activities. A few changes in the LAF assay are also proposed, namely the use of the MTT colorimetric method (Mosmann, 1983) in substitution to the radioactive uptake method for the assessment of celular proliferation, and of the lectin jacalin as the co-mitogenic lectin.

MATERIALS AND METHODS

Animals – Thymuses were obtained from male C3H/HeJ or Balb/c mice aging 4 to 10 weeks, bred in the animal facilities of the National Cancer Institute (Rio de Janeiro, Brazil).

Reagents – Culture medium, antibiotics, 2-mercaptoethanol (2-Me), L-glutamine, dimethylthiazol-diphenyltetrazolium bromide (MTT), phytohemagglutinins M (PHA-M, L-8887) P (PHA-P, L-8754), and P (PHA-P*, L-9017), and concanavalin A (Con A, C-2010), were purchased from Sigma Co., St. Louis, MO. Fetal calf serum (FCS) was obtained from Cultilab, Campinas, SP. [^{3}H]-thymidine (^{3}H TdR, 2 Ci/mmole) was purchased from Dupont, Boston, MA. Jacalin was prepared as previously described (Dalmau et al., 1989). IL-1 containing conditioned media were prepared by stimulating human mononuclear adherent cells with lipopolysaccharide in a nylon wool column (CM) or in a plastic culture flask (CM1) (Dalmau & Freitas, 1990b). Recombinant human interleukin 1β (sp. act. 10^6 units/mg of protein) and recombinant human interleukin 2 (sp. act. 10^6 units/mg of protein), both from Hoffman-LaRoche, Nutley, were kindly provided by Dr. Richard Peck, Basel Institute for Immunology, SW.

Pre-incubation of thymocytes – Thymocytes were adjusted to a final concentration of 3 x 10^7 viable cells/ml in RPMI 1640 medium plus 2 mM freshly added glutamine, 5% FCS, 5 x 10^{-5} M 2-Me (culture medium), with or without IL-2 and incubated in 25 cm^2 plastic bottles for 24 hr at 37 °C under a humidified atmosphere containing 5% CO_2. Cells were then pelleted by centrifugation, washed four times and once suspended in culture medium as in the original volume.

Thymocyte mitogenic assays – Thymocytes (1.5 x 10^6/well) were plated on 96 well flat bottom plates in final volumes of 0.2 ml (for the radioactive method) or 0.1 ml (for the MTT method) of culture medium. Cultures were run in triplicates for 48 to 96 hr, as indicated for each experiment. Tritiated thymidine (0.5 μCi/25 μl/well) was added for the last 18-24 hr of culture, and incorporation measured by liquid scintillation counting in a toluene-PPO-POPOP cocktail. The MTT method was carried out according to Mosmann (1983), in a 2 hr incubation with the substrate (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide; Sigma Chem. Corp.) in 10 ul of a 5 mg/ml stock solution in PBS, and the reaction stopped by the addition of 0.15 ml of 0.04 N HCl in isopropanol. Solubilization of formazan was accelerated by floating the plates for 1 min on an ultrasound water bath (Chubby, 25 megahertz, 900 watts; Thornton-Inpec Eletron, SA, SP).

RESULTS

Pre-incubation with IL-2 renders thymocytes more responsive to IL-1 and more sensitive to CM – In experiments performed to test the participation of different cytokines in thymocyte proliferation, we observed that the pre-incubation with IL-2 rendered murine thymocytes more responsive to IL-1 (unpublished). Thymocytes pre-incubated with IL-2 (50 u/ml) for 24 hr proliferated three to five fold more to IL-1 in the presence of PHA than did fresh or medium pre-incubated ones (Figs 1, 2, A, B). Interestingly, IL-2 pre-incubated thymocytes were found to be more sensitive (i.e. respond to lower amounts of IL-1 or CM) rather

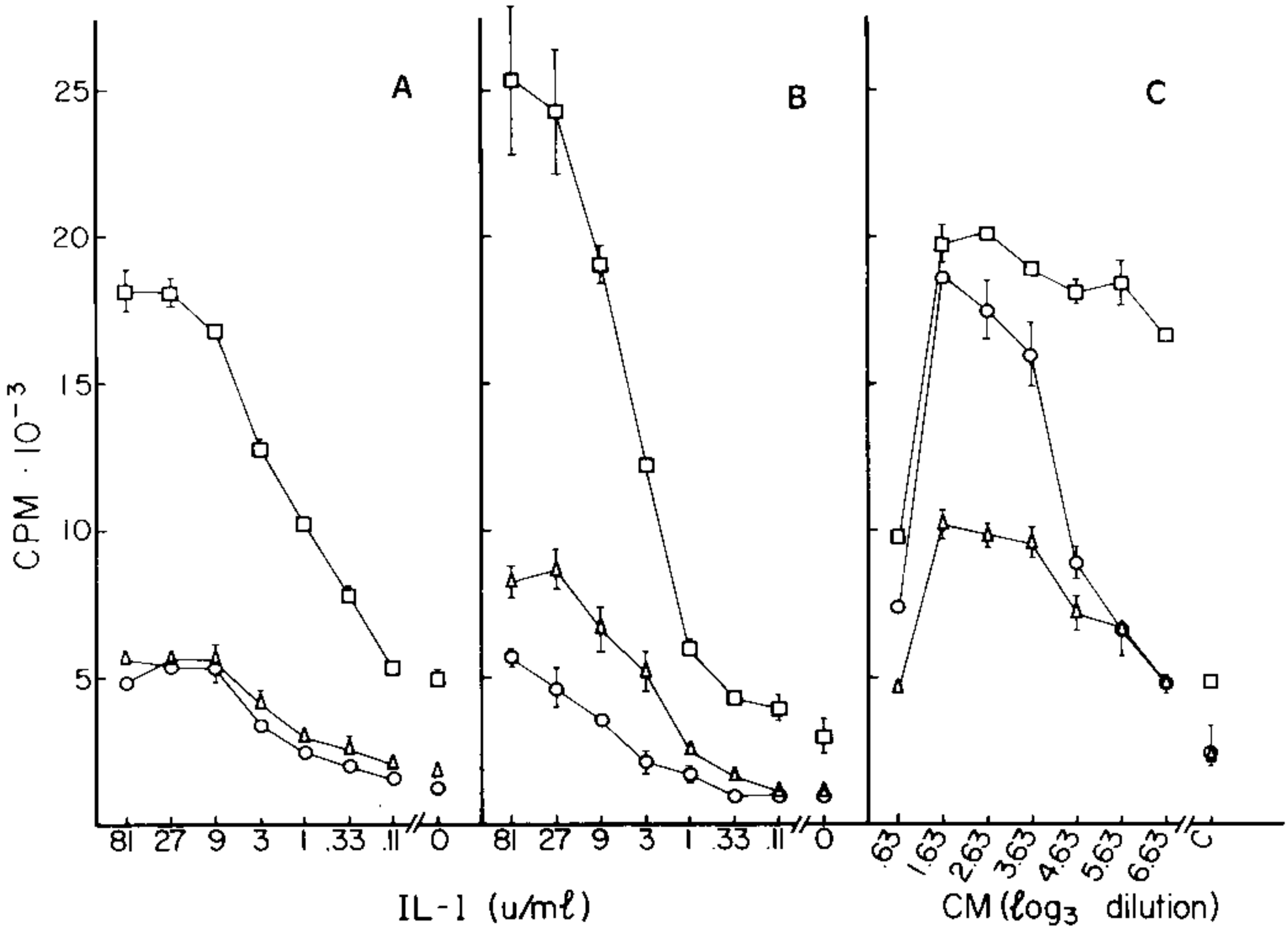

Fig. 1: effect of pre-incubation of C3H/HeJ thymocytes with IL-2 on their response to IL-1 or CM. Freshly prepared thymocytes (○) were seeded in microtiter plates and incubated for 72 (A and C) or 96 hr (B) in the presence of PHA-M (20 µg/ml) plus IL-1 (A and B) or plus CM (C), as in the classical LAF assay. Thymocytes from the same suspension were incubated without (△) or with (❑) 50 u/ml IL-2 for 24 hr, and then seeded in microtiter plates for additional 48 hr (A and C) or 72 hr (B) with PHA-M and IL-1. The ^{3}H TdR uptake time (last 24 hr of culture) was run simultaneously for the three conditions (○, △, ❑), with equal total periods of culture.

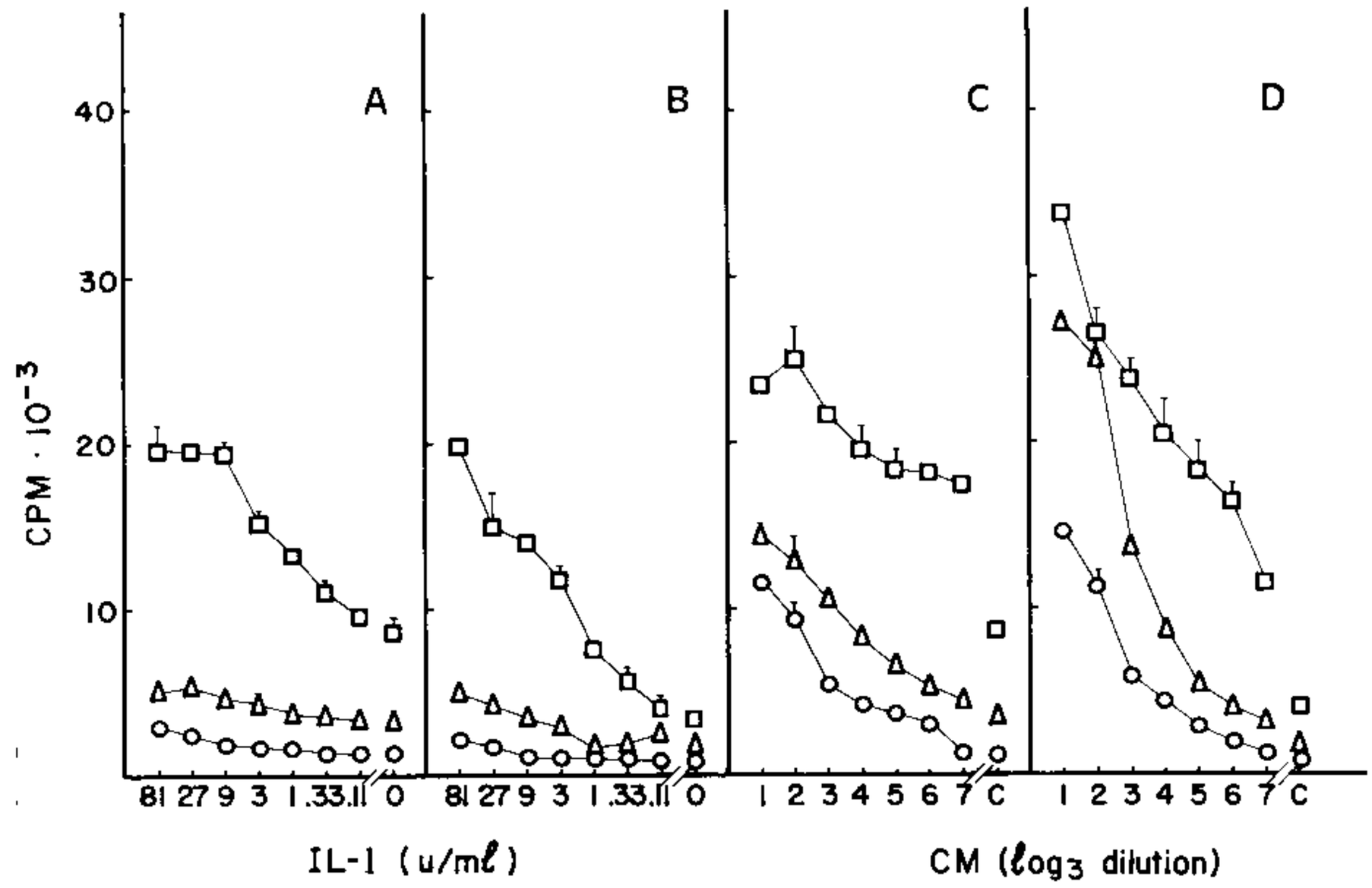

Fig. 2: effect of pre-incubation of murine Balb/c thymocytes with IL-2 on their response to IL-1 or CM. Freshly prepared thymocytes (○) were seeded in microtiter plates and incubated for 72 (A and C) or 96 hr (B and D) in the presence of PHA-M plus IL-1 (A and B) or plus CM (C and D), as in the classical LAF assay. Thymocytes from the same suspension were incubated without (△) or with (❑) 50 u/ml IL-2 for 24 hr, and then seeded in microtiter plates for additional 48 (A and C) or 72 hr (B and D). The ^{3}H TdR uptake time (last 24 hr of culture) was run simultaneously for the three conditions (○, △, ❑), with equal total periods of culture.

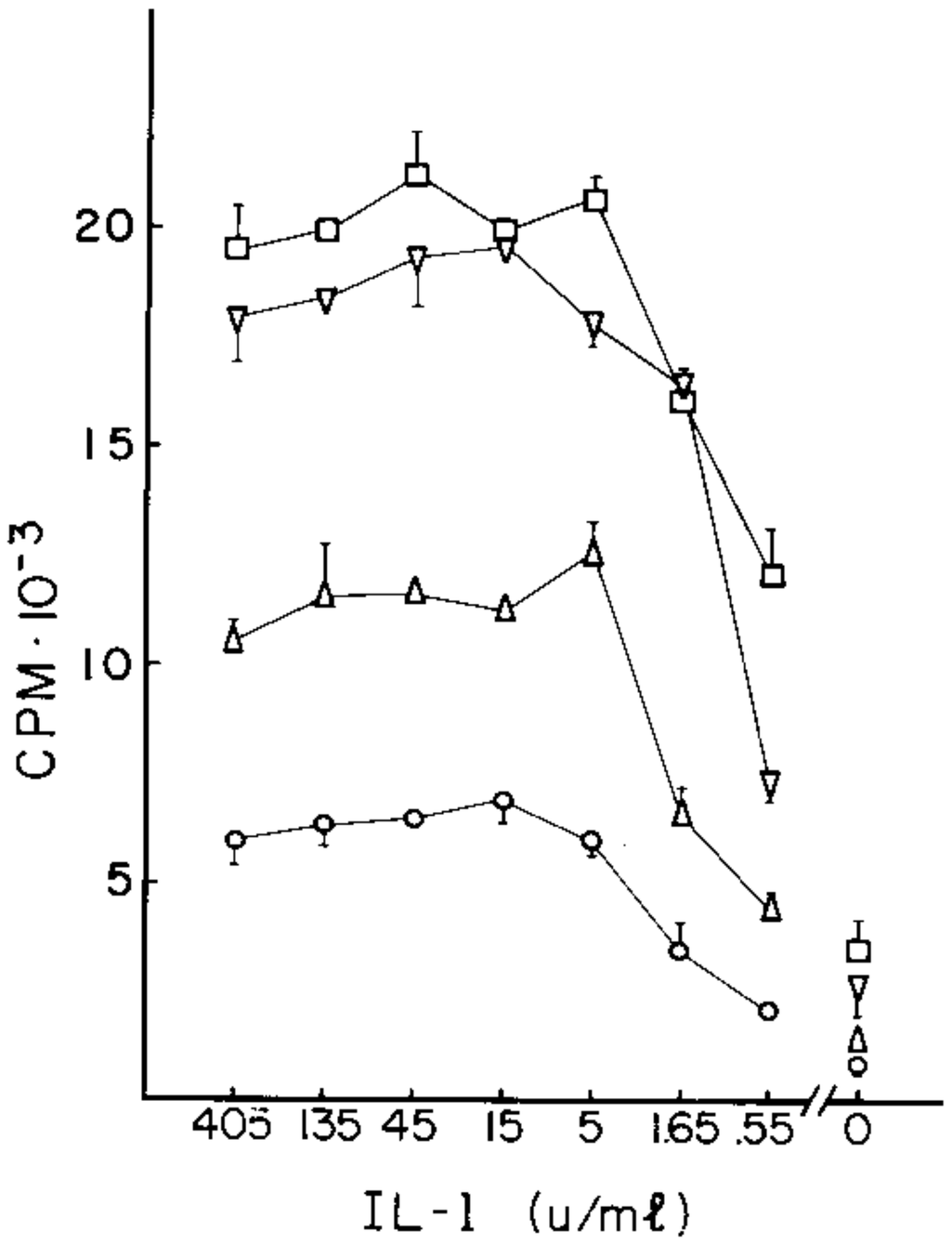

Fig. 3: effect of pre-incubating thymocytes with different doses of IL-2 on their response to IL-1. Thymocytes from C3H/HeJ mice were pre-incubated in the absence (○) or in the presence of 1 (Δ), 10 (Δ) or 100 u/ml IL-2 (□) for 24 hr and then assayed for additional 48 hr to their proliferative response to IL-1 in the presence of PHA-M.

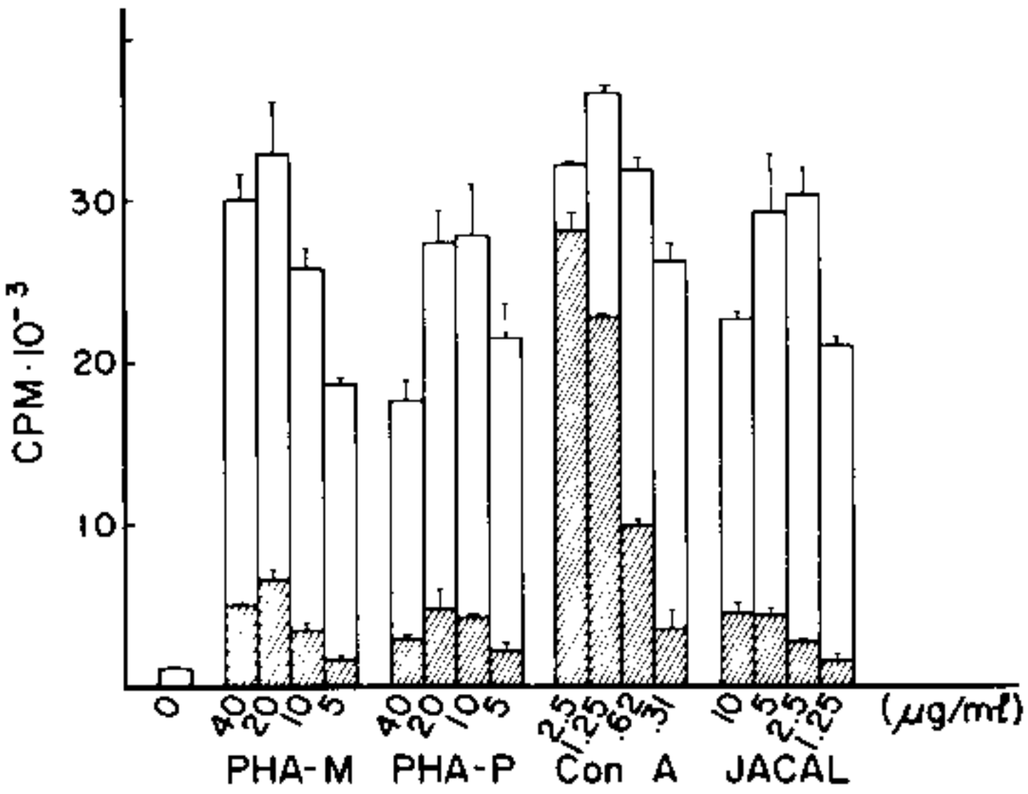

Fig. 4: mitogenic and co-mitogenic activities of different lectins in the LAF assay. C3H/HeJ thymocytes were stimulated with diverse lectins in multiple amounts in the absence (dashed bars) or in the presence (open bars) of CM (1/27 dilution) for 72 hr.

than more responsive (i.e. present a higher ^{3}H TdR uptake) to human monocyte CM (Figs 1C, 2C, 2D). Maximal enhancing effect on thymocyte responsiveness to IL-1 could already be reached with IL-2 doses near 10 u/ml (Fig. 3). Pre-incubation with IL-2 did not change the thymocyte sensitivity to PHA (not shown).

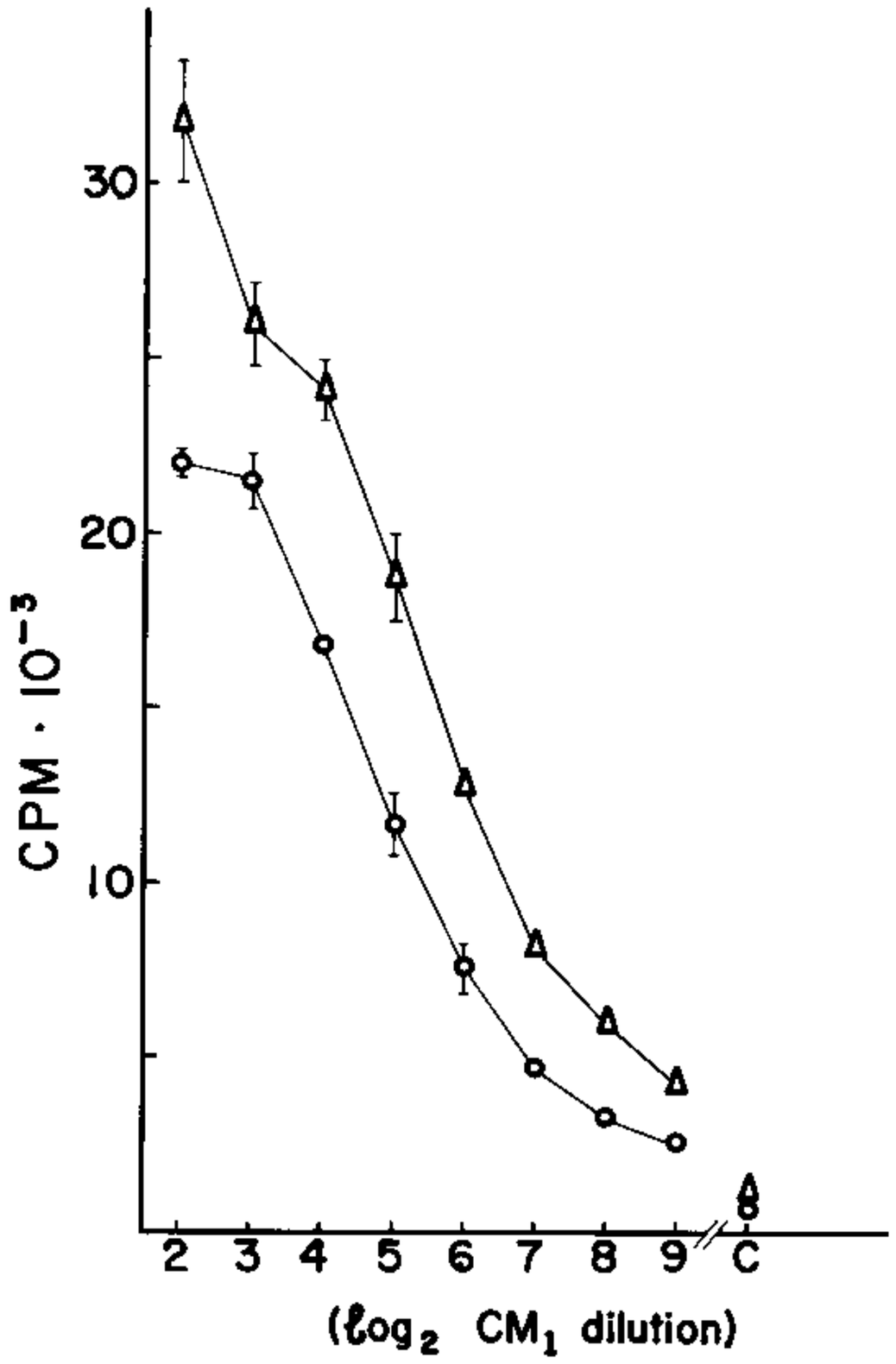

Fig. 5: use of jacalin in substitution to PHA in the LAF assay. C3H/HeJ thymocytes were layered over a titered conditoned medium (CM1) in the presence of 20 μg/ml PHA-M (○) or 2 μg/ml jacalin (Δ) and incubated for 72 hr.

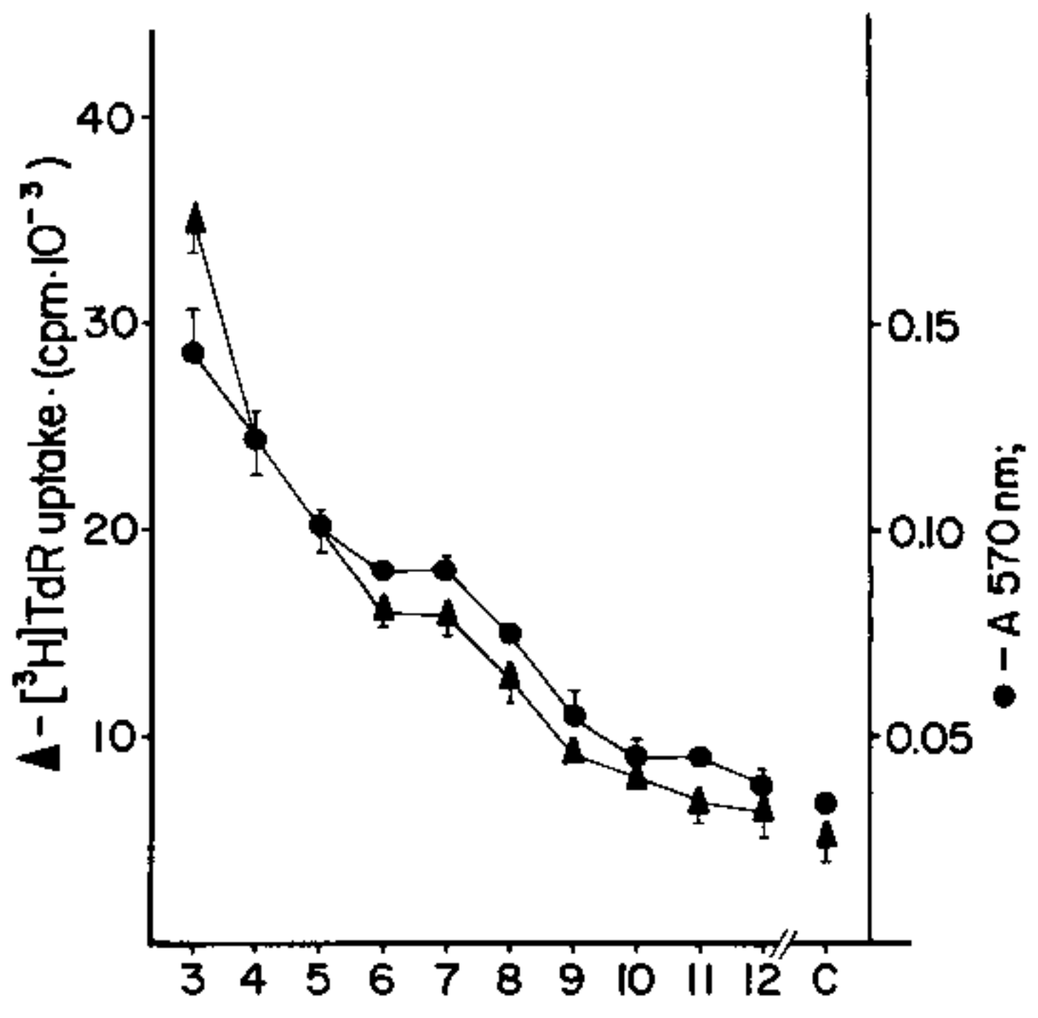

Fig. 6: correlation between ^{3}H TdR incorporation and MTT reduction in the measurement to thymocyte proliferation. C3H/HeJ thymocytes were stimulated with serial dilutions of CM1 in the presence of PHA-M for 72 hr. ^{3}H TdR and MTT were added respectively 24 hr and 2 hr prior to the end of cultures.

TABLE

Comparative data between the tritiated thymidine and the MTT colorimetric methods in the LAF assay

Lectin	Method	Controls (C)	Conditioned medium (CM)	C/CM x100
PHA-P	^{3}H TdR	2.730 (1032)	35.535 (1464)	7.7
	MTT	.010 (.002)	.453 (.016)	2.3
PHA-M	^{3}H TdR	1.055 (64)	25.445 (403)	4.1
	MTT	−.004 (0)	.380 (.013)	0
PHA[a]	^{3}H TdR	905 (35)	13.190 (85)	6.9
	MTT	.044 (.003)	.253 (.011)	17.4
Con A	^{3}H TdR	6.300 (933)	28.480 (4398)	22.0
	MTT	.070 (.004)	.350 (.015)	20.0

a: PHA, phytohemagglutinin Wellcome, Dartford, UK. Thymocytes were co-stimulated with the indicated lectins in the absence (controls) or the presence of CM (1/10) for 72 hr. For the radioactive method, ^{3}H TdR was added 24 hr before the end of culture and the results are shown in cpm. For the enzymatic method, the methyl tetrazolium was added 2 hr prior to the end of culture (the stop of reaction) and the results are shown as A_{570nm}, obtained by the use of an ELISA reader. Numbers between parenthesis represent the SD of the means of triplicates.

The use of jacalin and the MTT method in the LAF assay – A critical feature in the LAF assay is the choice of the lectin and its concentration. A suitable lectin should induce, at suboptimal or optimal amounts, satisfactory co-mitogenic response while keeping at low values its intrinsic mitogenic effect. Fig. 4 shows comparative results obtained with PHA-M, PHA-P, Con A, or jacalin, when tested for thymocyte co-stimulation with a CM diluted 1/27. As can be seen, jacalin is as good as PHA in its co-mitogenic capacity and exerts a similarly low mitogenic activity on thymocytes, conversely to Con A, that has a high mitogenic activity even at suboptimal doses. The suitability of using jacalin in this assay is shown in Fig. 5, which shows the titration curves of a conditioned medium (CM1) in the presence of either PHA (20 μg/ml) or jacalin (2 μg/ml).

The MTT reaction represents the activation of reduction pathways mainly at the mitochondrial level (Mosmann, 1983) what could potentially occur with non-dividing thymocytes exposed to lectins and/or cytokines. However, under phase contrast microscopy, only enlarged lymphocytes (blasts) and adherent thymic stromal cells presented in their cytoplasm formazan cristals (not shown). The suitability of the use of the MTT method in the LAF assay, and consequently in our proposed pre-IL-2 assay is shown in Fig. 6, in which a CM was titered in the presence of PHA and the response measured by both the MTT reduction and the ^{3}H TdR uptake methods. Table shows the results obtained when murine thymocytes were exposed to the indicated doses of phytohemagglutinins or Con A in the presence of a 1/10 diluted CM, and the responses assessed by the two methods.

DISCUSSION

The main advantage of the assay proposed by Falk et al. (1987) (co-IL-2 assay) is to allow the measurement of IL-1, and probably other cytokines, in samples carrying IL-2. In our hands this assay also proved to be more responsive to pure IL-1 than the LAF assay (not shown). However, one should be careful when measuring IL-1 activity in samples containing lectins, since in the presence of IL-2, thymocytes strongly proliferate even to poorly mitogenic lectins as PHA or jacalin (Carding & Bottomly, 1988; our unpublished data). The presently proposed pre-IL-2 assay increases the responsiveness of thymocytes to IL-1 to a magnitude similar to that of the co-IL-2 assay (not shown). As the classical LAF assay, it has the disadvantage of being sensitive to exogenous IL-2. Nonetheless, it allows the measurement of IL-1 in samples containing lectins such as PHA or jacalin.

Occasional high backgrounds (i.e. proliferation to lectin alone) when pre-IL-2 assay was run for 72 hr (24 hr of IL-2 pre-incubation plus 48 hr of incubation) can be overcome, as in the LAF assay, by extending the total time of the assay to 96 hr. This will increase the slope of the curves, allowing a more accurate unit determination, although decreasing the sensitivity of these assays. The same procedure is not effective when attempting to reduce the background in the co-IL-2 assay.

The mechanism(s) by which pre-incubation with IL-2 increases the thymocyte responsiveness for IL-1 or the sensitivity to CM remains to be determined. Interleukin 2 has been shown to have a very poor ability of inducing its own receptor on thymocytes in the absence of lectins (Hanson et al., 1983). An effect of IL-2 on the survival or expansion of IL-1 responsive cells can not totally explain the observed results,

since pre-incubation in the absence of IL-2 increases (although slightly) the responsiveness of thymocytes to IL-1, and flow cytometry analyses of CD4 and CD8 distribution on thymocytes pre-incubated either with or without IL-2 showed similar profiles (data not shown). Experiments aiming to detect differences in cytokine secretion by thymocytes after pre-incubation in the presence or absence of IL-2 are presently being carried out.

The choice of poorly mitogenic lectins such as PHA or jacalin, which can be used in the LAF assay even at optimal or overoptimal doses, is important to avoid significant changes in free lectin, once erythrocytes can variably contaminate the thymocyte preparations. More reproducible assays can be obtained with those lectins than with Con A, which should be used in a narrow sub-optimal dose range (usually 0.25 to 1 μg/ml, depending on the thymocyte preparation and mouse strain used (Dalmau & Freitas, 1990a), to achieve a good co-stimulatory effect with a poor background signal. The murine thymocyte co-stimulatory assays initially proposed for the measurement of IL-1 or IL-2 have already been substituted for more specific biological or immunochemical assays. However, they remain as useful tools for an initial cytokine search in biological samples. They can be used for the detection of natural cytokine inhibitors (Svenson & Bendtzen, 1988), immunostimulants (Dalmau et al., 1990), and when assaying human monocyte culture broths the LAF assay is quite specific for detecting IL-1 activity (Dalmau et al., 1991). In this context we think that the pre-IL-2 assay is more responsive or sensitive than the classical LAF assay in which the alterations herein suggested can also be incorporated.

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REFERENCES

CARDING, S. R. & BOTTOMLY, K., 1988. IL-4 (B cell stimulatory factor 2) exhibits thymocyte growth factor activity in the presence of IL-2. *J. Immunol., 140:* 1519-1526.

CONLON, P. J.; HENNEY, C. S. & GILLIS, S., 1982. Cytokine-dependent thymocyte responses: characterization of IL-1 and IL-2 target subpopulations and mechanism of action. *J. Immunol., 128:* 797-801.

CONLON, P. J.; MORRISSEY, P. J.; NORDAN, R. P.; GRABSTEIN, K. H.; PRICKETT, K. S.; REED, S. G.; GOODWIN, R.; COSMAN, D. & NAMEN, A. E., 1989. Murine thymocytes proliferate in direct response to interleukin-7. *Blood, 74:* 1368-1373.

DALMAU, S. R. & FREITAS, C. S., 1990a. Standardization of the IL-1 comitogenic assay using thymocytes from LPS-sensitive mouse strains. *Mem. Inst. Oswaldo Cruz, 85:* 123-125.

DALMAU, S. R. & FREITAS, C. S., 1990b. Nylon wool columns- a tool for obtaining monokine-rich eluates in the absence of serum. *Mem. Inst. Oswaldo Cruz, 85:* 153-162.

DALMAU, S. R.; FREITAS, C. S.; VERICIMO, M. A. & TORRES, S. T., 1990. Nigrosin dye promotes murine thymocyte proliferation. *Med. Sci. Res., 18:* 267-268.

DALMAU, S. R.; MACIEL, C. M. & FREITAS, C. S., 1989. Jacalin: an excellent lectin for obtaining T cell growth activity from rat spleen cells. *Braz. J. Med. Biol. Res., 22:* 1111-1120.

DALMAU, S. R.; SABINO, K. C.; PREZA, P. C. & FREITAS, C. S., 1991. Human monokine participation in bulk murine thymocyte participation. *Mem. Inst. Oswaldo Cruz, 86:* 501-503.

ELIAS, J. A.; TRICHIERI, G.; BECK, J. M.; SIMON, P. L.; SEHGAL, P. B.; MAY, L. T. & KERN, J. A., 1989. A synergistic interaction of IL-6 and IL-1 mediates the thymocyte stimulating activity produced by recombinant IL-1-stimulated fibroblasts. *J. Immunol., 142:* 509-514.

FALK, W.; KRAMMER, P. H. & MANNEL, D. N., 1987. A new assay for interleukin-1 in the presence of interleukin-2. *J. Immunol. Methods, 99:* 47-53.

FALK, W.; MANNEL, D.; DARJES, H. & KRAMMER, P., 1989. IL-1 induced high affinity IL-2 receptor expression of $CD\text{-}4^{-}$ $CD8^{-}$ thymocytes. *J. Immunol., 143:* 513-522.

GERY, I.; GERSHON, R. K. & WAKSMAN, B. H., 1972. Potentiation of the T-Lymphocyte response to mitogens. I. The responding cell. *J. Exp. Med., 136:* 128-142.

GOTO, M.; FUJISAWA, M.; YAMADA, A.; OKABE, T.; TAKAKU, F.; SASANO, M. & NISHIOKA, K., 1990. Spontaneous release of angiotensin converting enzyme and interleukin 1β from peripheral blood monocytes from patients with rheumatoid arthritis under a serum free condition. *An. Rheum. Dis., 49:* 172-180.

HANSON, D. F.; MURPHY, P. A.; SILICANO, R. & SHIN, H. S., 1983. The effect of temperature on the activation of thymocytes by interleukins I and II. *J. Immunol., 130:* 216-222.

HELLE, M.; BRAKENHOFF, J. P. J.; DEGROOT, E. R. & AARDEN, L. A., 1988. Interleukin 6 is involved in interleukin 1-induced activities. *Eur. J. Immunol., 18:* 957-959.

LOWENTHAL, J.; BALLARD, D.; BOGERD, H.; BOHNLEIN, E. & GREENE, W., 1989. Tumor necrosis factor α activation of the IL-2 receptor α gene involves the induction of k B-specific DNA binding proteins. *J. Immunol., 142:* 3121-3132.

MANNEL, D. N.; MIZEL, S. B.; DIAMANTSTEIN, T. & FALK, W., 1985. Induction of interleukin 2 responsiveness in thymocytes by synergistic action of interleukin 1 and interleukin 2. *J. Immunol., 134:* 3108-3114.

MOSMANN, T. R., 1983. A rapid colorimetric assay

for cellular growth and survival: application to proliferation and cytotoxic assays. *J. Immunol. Methods, 65:* 55-62.

OKASAKI, H.; ITO, M.; SUDO, T.; HATTORI, M.; KANO, S.; KATSURA, Y. & MYNATO, N., 1989. IL-7 promotes thymocyte proliferation and mantains immunocompetent thymocytes bearing αβ or τδ T-cell receptors *in vitro:* synergism with IL-2. *J. Immunol., 143:* 2917-2922.

RANGES, G. E.; ZLOTNIK, A.; ESPEVIK, T.; DINARELLO, C. A.; CERAMI, A. & PALLADINO Jr., M. A., 1988. Tumor necrosis factor α/cachectin is a growth factor for thymocytes. Synergistic interactions with other cytokines. *J. Exp. Med., 167:* 1472-1478.

SABINO, K. C.; DALMAU, S. R. & PREZA, P. C. A., 1989. Mononuclear proliferation and IL-1 secretion in rheumatoid arthritis (RA). *Proceedings of the* 7th Intern. Congress of Immunology, Berlin, Abstr. 83-61.

SHAW, J.; MONTICONE, V. & PAETKAU, V., 1978. Partial purification and molecular characterization of a lymphokine (costimulator) required for the mitogenic response of mouse thymocytes *in vitro. J. Immunol., 120:* 1967-1973.

SUDA, T.; MURRAY, R.; GHIDOS, C. & ZLOTNIK, A., 1990. Growth-promoting activity of IL-1α, IL-6, and tumor necrosis factor-α in combination with IL-2, IL-4, or IL-7 on murine thymocytes. Differential effects on CD4/CD8 subsets and on $CD3^+/CD3^-$ double-negative thymocytes. *J. Immunol., 144:* 3039-3047.

SVENSON, M. & BENDTZEN, K., 1988. Inhibitor of interleukin 1 in normal human urine. Different effects on mouse thymocytes and on a murine T-cell line. *Scand. J. Immunol., 27:* 593-599.