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Regulatory T-cell subpopulations in pregnancy

II. EVIDENCE FOR SUPPRESSIVE ACTIVITY OF THE LATE PHASE OF MLR

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Summary. Adding mitomycin-treated spleen cells from an allopregnant mouse to a maternal-strain cells *v.* paternal-strain cells MLR was previously shown to decrease the intensity of the reaction, due to suppressor T cells. In the present study, spleen cells from allogeneically pregnant mice, were added without treatment at day 2 of an MLR of maternal strain responder cells raised against paternal-strain stimulators. It was shown that, while, on 2 day duration culture versus the same stimulator cells these cells are hyper-reactive compared to controls, yet they lead to a MLR of lower intensity than when the same operation was performed with control cells. This suppressive effect is T-cell dependent and use of high dilutions of anti-Thy 1+C suggests that the suppressor cell has a high density of Thy 1 surface antigen. Involvement of these cells in negative regulation of the late phase of the MLR is suggested. MLRs with cells from allopregnant mice as responders were themselves susceptible to this suppression and even more so than cells from virgin mice, indicating a possible physiological role.

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INTRODUCTION

We have previously reported evidence for suppressor T-cell activity in the spleens of multiparous mice undergoing their second week of allogeneic gestation. *In vivo*, suppressor T cells are able to enhance the growth of a tumour graft from paternal-strain origin on maternal-strain recipients, (Chaouat, Voisin, Daëron & Kanellopoulos, 1977; Chaouat, Voisin, Escalier & Robert, 1979), or, as reported by others, male skin graft in the B10.HY system (Smith & Powell, 1977). *In vitro*, it was shown that, added at the onset of a MLR of maternal-strain splenocytes raised against paternal-strain stimulators, they could lead to a marked suppression both in total c.p.m. and stimulation indexes. Data to be published from this laboratory, and a report by an independent group (Clark & McDermott, 1978) suggest these cells could also impair CTL generation by the mother against her conceptus, as reported also by Hamilton & Hellström (1977). Our first assay system used a protocol similar to the system of Rich & Rich (1974) first described for the study of MLR regulatory mechanisms after a transplantation allosensitization. It involved mitomycin-C pre-treatment of the cell population later assayed for putative suppressive activity (Chaouat & Voisin, 1979). Little is yet known about the possible differential effects of such a drug upon various T-cell subsets, and in altered

hormonal conditions as is the case during pregnancy. It has even been shown that suppressor activity of cells from lymph nodes of allosensitized animals was lost after mere 24 h culture, while being protected, and even enhanced, precisely after mitomycin C treatment (Wagner, Starzinski-Powitz, Pfizenmaier & Rollinghoff, 1976). Since the reasons for such a discrepancy between the outcome of the two protocols remain unclear, it appeared necessary to study MLR regulatory effects of untreated cells, to get rid as far as possible of any drug-induced artefact. The present paper depicts the results of such an investigation.

MATERIALS AND METHODS

Animals

CBA/J (H-2^k), C57Bl/Ks (H-2^d), A/J(H-2^a) or CBA-Ca Iffa (H-2^k) were inbred in our laboratory or purchased from IFFA-CREDO, l'Arbresle, France. Animals used for the supply of responder and stimulatory cells were 8–14 weeks old. Cells from animals tested for putative regulatory activity were collected from CBA/J undergoing their third or fourth allo or isopregnancy, around the second week of gestation as estimated by both vaginal plug recording and litter size and morphological development. Virgin CBA ♀ of the same age were used as controls.

MLR culture conditions

The basic procedure has been detailed elsewhere (Chaouat & Voisin, 1979). It includes a slight modification of Peck & Bach (1973) technique. Briefly, $6{\cdot}5 \times 10^5$ responder cells were mixed with $6{\cdot}5 \times 10^5$ mitomycin treated cells, in a total volume of $2 \times 100\mu l$ (or sometimes $2 \times 125\mu l$), in Linbro flat-bottomed microculture plates. The standard culture medium used was RPMI–1640 (GIBCO Biocult Inc., or Biopro) supplemented with 2 mM HEPES buffer, 10U/ml penicillin streptomycin solution, 1 mM sodium bicarbonate and $2{\cdot}5 \times 10^{-5}$ M mercaptoethanol, with 1% heat-inactivated (30 min, 56°) normal mouse serum. Cultures were maintained at 37° in a 10% CO_2 humid atmosphere. At day 2, 100 μl of regulatory cells suspension ($6{\cdot}5 \times 10^6$ cells/ml), prepared in the same culture medium, were added to the wells. Except when specifically quoted (see kinetic studies) 10μl of [^{3}H]-thymidine were added on day 3, and cells collected after 24 h of radioactive pulse, on Whatman GF-A glass fibre filters, using a multiple automatic sample harvester (MASH). After 1 h drying at 37°, samples were put into 2 ml of POPOP scintillation fluid, and c.p.m. measured using a SL 30 scintillation counter (Intertechnique, France). Results are expressed as mean c.p.m. (minus background) $\pm$ SD of the mean of at least three replicates (usually four). Stimulation indexes of both test and regulated cultures were computed by referring to c.p.m. of isogeneic mixture combination (syngeneic MLR) started on day 0. However, addition of untreated cells at day 2 obviously alters also day 4 'background' c.p.m. Therefore, a 'relative response index' was also computed as follows: RRI = (day 4 c.p.m. of 'regulated' allogeneic culture)/(day 4 c.p.m. of 'regulated' syngeneic culture).

Statistical analysis

This was computed using two sample Student's t test on c.p.m. value of replicates from each experiment, or paired Student's t test on stimulation indexes of each assay.

RESULTS

Effect of addition of splenocytes from allogeneically pregnant mice

Untreated putatively regulatory cells were added to 48 h old MLRs for the following reasons. (a) To minimize the [^{3}H]-thymidine uptake by the regulatory cell population as a consequence of *in vitro* rechallenge by alloantigenic cells (as compared to what it would have been, would the addition have been made on day 0). Such a reactivity could mask a suppressive activity upon the test MLR, also assessed by [^{3}H]-thymidine incorporation, and then confuse the interpretation, since it is theoretically possible, as in the Tse and Dutton system (Tse & Dutton, 1977) to ascribe ^{3}H incorporation to Ly 2, 3 cells proliferating as bystander of suppressor cells themselves. (This was also judged not a theoretical possibility in view of the kinetics results reported in our previous paper.) (b) To minimize a possible loss of suppressive activity in absence of mitomycin treatment, in a way similar to the observations described by Wagner *et al.* (1976). Such a system requires scrutiny of the results at three levels: (a) stimulation index (b) effect on the syngeneic culture itself (c) relative response index.

Effect on the stimulation index. In about 3/4 of the experiments, a profound suppression was observed, lowering the c.p.m. of the MLR regulated by cells from allopregnant mice, and thus the stimulation index, below the test (not regulated) MLR itself (Table

Table 1. Effect of the addition of $6{\cdot}5 \times 10^5$ cells from virgin, syngeneically or allogeneically pregnant mice on CBA *v.* C57Bl/Ks MLR ($H\text{-}2^k$ *v.* $H\text{-}2^d$)

Cells incubated on day 0		Regulatory cells added at day 2 (untreated)	Counts per minute ± SD on day 4 (four replicates)	Stimulation index	Percentage of suppression	Relative response index
Stimulator (mitomycin-treated)	Responder					
C57Bl/Ks	Virgin CBA ♀	None	9,413 ± 1872	5·46		
		Virgin CBA ♀	14,511 ± 1078	8·42	—	2·97
		CBA ♀ pregnant from CBA ♂	13,998 ± 902	8·12	—	3·11
		CBA ♀ pregnant from A/J ♂	5,213 ± 332	3·02	72%	1·54
	None	Virgin CBA ♀	5,078 ± 293	—	($P < 10^{-4}$)	
		CBA ♀ pregnant from CBA ♂	4,862 ± 311	—		
		CBA ♀ pregnant from A/J ♂	9,366 ± 422	—		
CBA	Virgin CBA ♂	None	1,723 ± 210	—	—	—
		Virgin CBA ♂	4,873 ± 260	—	—	—
		CBA ♀ pregnant from CBA ♂	4,487 ± 349	—	—	—
		CBA ♀ pregnant from A/J ♂	3,385 ± 263	—	—	—

Table 2. Comparison in the same experiment of the suppressive effect of spleen cells from allogeneically pregnant mice of the MLR of cells from virgin, syngeneically or allogeneically pregnant mice, taken at second week of pregnancy

Cells incubated on day 0		Regulatory cells added at day 2 (or 'responders' when alone with stimulators)	Counts per min ± SD and ( ) mean c.p.m. of 'regulated syngeneic MLR' (mean of four replicates)	Stimulation index and ( ) relative response index	Percentage of suppression	Statistical significance $P <$
Stimulator (Mitomycin-treated)	Responder					
A/J m	CBA♀ virgin	—	1,965 ± 289·0	2·57		
		CBA ♀ pregnant from CBA ♂	3,541 ± 150·0 (1650)	4·72 (2·14)	20%	0·05
		CBA ♀ pregnant from A/J ♂	2,926 ± 146·0 (1520)	3·88 (1·92)		
		Virgin CBA ♀	3,725 ± 261·0 (1715)	4·98 (2·17)	22%	0·01
A/J m	CBA ♀ pregnant from CBA ♂	—	1,251 ± 167·0	1·88		
		CBA ♀ pregnant from CBA ♂	3,260 ± 622·0 (1565)	4·90 (2·08)	18%	10^{-2}
		CBA ♀ pregnant from A/J ♂	2,798 ± 238·0 (1470)	4·19 (1·90)		
		Virgin CBA ♀	3,768 ± 712·0 (1590)	5·68 (2·36)	34%	10^{-3}
A/J m	CBA ♀ pregnant from A/J ♂	—	2,488 ± 224·5	2·08		
		CBA ♀ pregnant from CBA ♂	4,102 ± 60·0 (1996)	3·48 (2·05)	56%	10^{-3}
		CBA ♀ pregnant from A/J ♂	2,608 ± 440·0 (1773)	2·19 (1·47)		
		Virgin CBA ♀	3,745 ± 608·0 (1887)	3·17 (1·99)	53%	10^{-3}
A/J m	None (CBA m)	CBA ♀ pregnant from CBA ♂	2,215 ± 213·0	—	—	
		CBA ♀ pregnant from A/J ♂	2,825 ± 293·0	—	—	
		Virgin CBA ♀	1867·5 ± 237	—	—	

1). In about 1/4 of the experiments, however, as shown in Table 2, suppression was less intense, and apparent only by the fact that c.p.m. and stimulation indexes of MLRs with added cells from allogeneically pregnant mice were below those of control cultures (e.g. cultures with added cells from isogeneically pregnant or virgin animals). It should be stressed that, as already reported (Chaouat & Voisin, 1979), the c.p.m. of a 2 day culture of cells from allopregnant mice versus mitomycin treated stimulatory cells (put at day 0 into culture wells) were always higher than similar responses of cells harvested from control animals (see Tables 1 and 2). Similar results were also obtained with cells responding with fresh stimulators set on day 2 into culture wells, or with cells responding against a 1 : 1 mixture of mitomycin-treated stimulator cells and splenocytes from responder strain set up at day 0 (data not given). The pattern of suppression observed was not correlated with the origin of the stimulator cells (e.g. H-2^d leading to high, or H-2^a, to low stimulation) nor with the one of the allopregnant cells (e.g. whether CBA ♀ had been mated with A/J or C57Bl/Ks♂).

Effect on the syngeneic (background) culture. As would have been easily predicted, addition of live cells at day 2 produced a marked increase of day 4 c.p.m. This was not due to feeding alterations, since addition of 50, 75, or 100 μl of fresh culture medium did not result in such an effect. However, (despite almost equal survival of cells cultured alone as compared to cells cultured with syngeneic mitomycin-treated cells during 2 days) the addition of cells from allopregnant mice resulted in 13/18 experiments in a lowering of day 4 c.p.m. in the syngeneic mixture compared with the same in presence of control cells, and in only three cases in a statistically significant increase (paired t test P value of eighteen experiments yielding $P < 0{\cdot}01$).

Effect on the relative response index. The c.p.m. of the allogeneically regulated culture being always lower than those of the control does *not* necessarily imply that the response was also altered in such a way when assessed by relative response indexes. Indeed, as would be expected, differences are less intense than when computed by stimulation indexes, but, with the exception of one culture, always in favour of a suppression by cells from allogeneically pregnant mice (paired t test giving $P < 0{\cdot}001$). (It should also be quoted that the c.p.m. of the allogeneically pregnant cell regulated culture were below the predicted c.p.m. that one would expect by summing up c.p.m. of the test culture + c.p.m. of the regulatory cells responding for 48 h versus the same target. Paired t-test analysis of predicted and experimental values yields $0{\cdot}01 > P > 0{\cdot}001$).

Kinetics

Addition of live cells and fresh medium might have resulted in a differential shift of the kinetics of the cultures according to the origin of the regulatory population. Therefore, cultures were set up and, after addition of regulatory populations on day 2, harvested at day 3, 4, 5, 6 respectively (and in one case also at day 7). Such an operation yielded two kinds of results (a) the viability of the 'regulated' culture was still significant till day 6 and even day 7, whereas viability of 'untreated' replicates was not assessable by [^{3}H]-thymidine incorporation beyond day 5 in 1% mouse serum condition (data not given). (b) In all cases, the suppression assessed by relative response indexes, was evident as early as day 3 till day 6 and even 7 (Fig. 1).

Differential effect according to the origin of responder cells

In order to investigate if such a suppressive mechanism

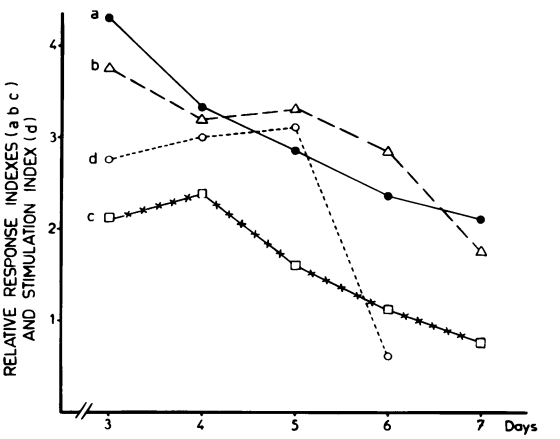

Figure 1. Kinetics of the suppression by spleen cells from allogeneically pregnant mice. Spleen cells were added on day 2 of a MLR of CBA responder cells versus mitomycin treated A/J stimulators. Cells added on day 2 came from: (a) virgin CBA female, (b) isopregnant CBA female and (c) CBA female pregnant of C57Bl/Ks (allopregnant). Results are expressed as 'relative response' (i.e. stimulation indexes computed against the similarly treated syngeneic MLR). Curve (d) gives, as a reference, the kinetics of the untreated MLR (without any cell addition and therefore expressed as a simple stimulation index).

could be operative during pregnancy, cells from allopregnant mice or control animals were added to cultures of responder cells from allopregnant or isopregnant mice. Suppression was also observed in such a case. It was always more marked on cells from allopregnant mice than control ones. This difference is especially noticeable in cases where, as shown in Table 2, the suppressive activity is mild and only apparent in c.p.m. and relative response indexes. Similarly (data not given) splenocytes from animals alloimmunized against stimulatory cells *in vivo* prior to the MLR (secondary reaction) were more prone to this type of suppression than cells from intact mice.

Abolition of the suppressive effect by anti-Thy 1 treatment

To seek T dependence of the suppressive effect, aliquots of 2 ml of cells from virgin, isogeneically or allogeneically pregnant mice, at 10×10^6 cells/ml, were incubated with optimal (1/15 or 1/30 final dilution) anti-Thy 1·2 for 30 min at 37°. They were subsequently washed and resuspensions of 2 ml in 1/10 guinea-pig complement (previously absorbed on splenocytes and thymocytes) further incubated 30 min at 37°. After two washes, cells were resuspended in 3·1 ml of culture medium, e.g. equivalent to an initial concentration of $6{\cdot}5 \times 10^5$ cells/ml.

After this treatment, the suppressive effect was no longer detectable (Table 3), although still demonstrable after either heat-inactivated anti-Thy 1.2 alone or C′ alone. Thus, suppression is dependent upon the presence of T cells from allopregnant mice.

One should also notice that anti-Thy 1.2 + C treatment simultaneously inhibits the reactivity of the added cell population, reducing the total reactivity from 18·25 (SI) to 13·74 in the case of cells from virgin CBA mice and from 19·34 to 14·00 in the case of cells from syngeneically pregnant mice, a fact that gives even more significance to the results observed with cells from allopregnant mice.

Differential effects of anti-Thy 1 on subsets of the regulatory population

Calibration of anti-Thy 1.2: activity at high dilution (see Fig. 2). At the same cell dilutions and resuspensions as above, titration of the complement-dependent cytotoxic activity of an anti-Thy 1.2 (AKR anti-C3H) has been performed by trypan blue exclusion test on cells from CBA mice. Low dilutions killed 100% of thymocytes and 45% of spleen cells. No more cytotoxic activity was detected at 1/600 dilution (Fig. 2). No difference was observed whether the CBA target cells reacting against A/J mitomycin-treated cells in a 4 day MLR came from normal mice or from mice pregnant of A/J. The [^{3}H]-thymidine incorporation levels, however and the stimulation indexes, were found to be differentially affected by high dilutions. As expected, the activity (SI) of reactive cells from virgin CBA mice *regularly* decreased, when increasing the anti-Thy 1.2 titre from 1/600 to 1/15, from optimal stimulation index with a plateau with anti-Thy 1.2 dilutions from 1/600 to 1/200. On the other hand, the reactivity of cells from allopregnant mice did not display such a pattern, in that 1/400 and 1/300 dilutions treatment resulted in an increase of stimulation indexes, before a regular decrease was again observed (Fig. 2). At this dilution of 1/400, only 10% of splenocytes and 17% of thymocytes were killed. The same effect was noted in CBA *v.* C57Bl/Ks MLRs when CBA cells came from females pregnant of C57Bl/Ks males (13,730 ± 340 c.p.m. after treatment of CBA cells with 1/400 anti-Thy 1.2 + C, as opposed to 11,367 ± 761, untreated).

High Thy 1 density of the suppressor cell population. The preceding results suggested selective depletion of suppressor cells by anti-Thy 1.2 at high dilutions (they did not result from passive enrichment in alloreactive cells since cells were resuspended in the same volume). The high dilution (1/400) treatment was then applied to aliquots of CBA cells either from control animals (syngeneically pregnant or virgin mice) or from experimental ones (allopregnant of A/J), before addition to the culture wells at day 2, as outlined above.

Table 4 shows the results of such a protocol applied to a MLR of CBA *v.* C57Bl/Ks regulated by cells from CBA females pregnant of A/J males. As can be seen the treatment with anti-Thy 1.2 (1/400) plus C abolished the suppression (difference between line 6 and line 9) *without* affecting the added reactivity brought by the cells added at day 2 (difference between line 1 and line 2 as well as 9). Control treatments such as anti-Thy 1.2 alone, C alone (and normal mouse serum + C, as verified in a separate experiment) remained without effect. This type of experiment was repeated four times with similar results. In addition, the same protocol, applied to regulatory cells from CBA females pregnant of C57Bl/Ks males, yielded similar results (two experiments).

In no case did anyone of the 2 anti-Thy 1.2 + C protocols result in an enhancement of the reactivity of

Table 3. Abolition by anti-θ‡ + C of the suppressive effect of spleen cells from allogeneically pregnant mice

MLR	Origin of cells added on day 2 (6×10^5)	Treatment of added cells	Counts per min ± SD and ( ) mean c.p.m. of 'regulated syngeneic MLR' (mean of three replicates)	Stimulation index and ( ) relative response index	Percentage of suppression*	Statistical significance $P <$
CBA *v.* (CBA) m	—	—	772 ± 17·95	—		
CBA *v.* (C57Bl/Ks) m	—	—	9,252 ± 1239	11·97	—	
	Virgin CBA ♀	—	14,101 ± 761 (2872)	18·25 (4·90)		
	CBA ♀ pregnant from CBA ♂	—	14,938 ± 730 (2653)	19·34 (5·63)	−0·12%	
	CBA ♀ pregnant from A/J ♂	—	6,873 ± 336 (2328)	8·89 (2·90)	55%	10^{-2}
						10^{-3}
CBA *v.* (C57Bl/Ks) m	Virgin CBA ♀	Anti-θ + C	10,613 ± 553 (1472)†	13·74 (7·21)†	—	10^{-4}
	CBA ♀ pregnant from CBA ♂	Anti-θ + C	10,816 ± 435 (1513)†	14·00 (7·14)†	0%	
	CBA ♀ pregnant from A/J ♂	Anti-θ + C	11,276 ± 352 (1328)†	14·59 (8·49)†	0%	

* Percentage of suppression is calculated by comparing with the addition of untreated virgin CBA cells.

† c.p.m. of 'syngeneic MLRs' (as in line 1) 'regulated' by anti-θ + C treated cells and relative response indexes computed by using this background.

‡ Anti-Thy 1.2 (AKR anti-C3H).

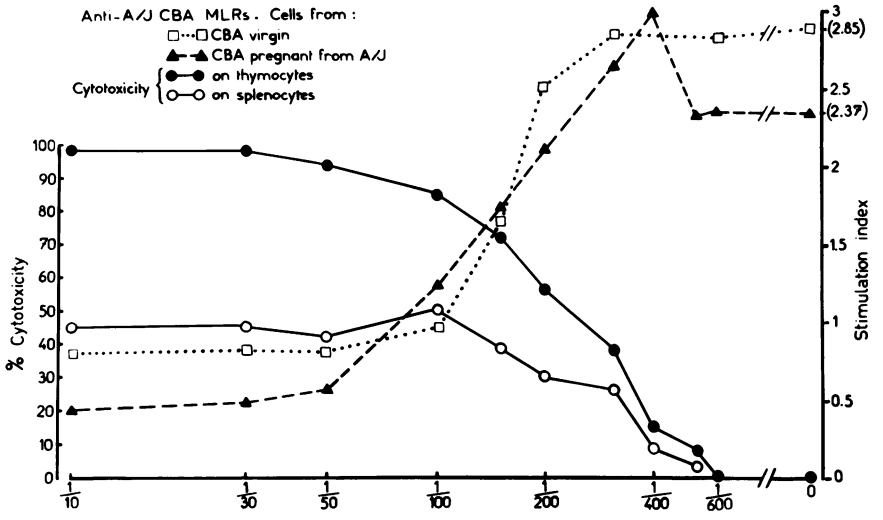

Figure 2. Effect of dilutions of anti-Thy-1 serum plus complement on lymphocyte viability and MLC reactivity. Cytotoxicity was assessed on thymocytes (●) or splenocytes (○) (left hand scale expresses the percentage of dead cells). MLC reactivity against A/J stimulators of spleen cells from virgin CBA(□) or CBA pregnant of A/J (▲) is measured by stimulation index at day 4 (right hand scale).

Table 4 Effect of suboptimal anti-θ* dilutions + C on the suppressive effect of allogeneically pregnant mice splenocytes

Origin of cells added on day 2† (6×10^5)	Treatment of added cells	Stimulation index	and statistical significance
None	—	5·27	
Virgin CBA ♀	None	7·44	↑
	Anti-θ alone	7·32	NS ↓
	C alone	7·68	↑
	Anti-θ + C	7·76	↓ $P < 10^{-4}$
			$P < 10^{-4}$ ↑
CBA ♀ pregnant from A/J ♂	None	3·67	↓
	Anti-θ alone	3·49	↑ $P < 10^{-4}$
	C alone	3·95	
	Anti-θ + C	7·69	↓

* Anti-Thy 1.2 (AKR anti-C3H), used at 1/400 dilution.

† The test MLC (to which the tested cells were added at day 2) was CBA *v.* C57Bl/Ks (H-2^k *v.* H-2^d) as in Table 3.

the MLR itself. It merely equalized the reactivity to that of the controls. This is in contrast with mitomycin-treated (early acting) regulatory cells of the previously described system (Chaouat & Voisin, 1979), where optimal doses of anti-Thy 1.2 + C lead to up to 30% enhancement of the MLR (in terms of c.p.m.), whereas use of suboptimal doses can lead from 80 to 200% enhancement (unpublished data).

DISCUSSION

The present results confirm our previous conclusions (Chaouat & Voisin, 1979) that it is possible to affect a MLR using cells allosensitized *in vivo* by multipregnancy, and extend them to the use of untreated regulatory populations added at day 2. Indeed, such an operation did result in marked decrease in c.p.m. reac-

tivity and stimulation indexes, when compared to the result of the equivalent operation performed with cells from virgin or isogeneically pregnant animals.

Furthermore, at least in the CBA *v.* C57Bl/Ks system, the reactivity of the MLR regulated by those cells was below the c.p.m. calculated by mere addition and even below the ones of the control culture itself. It can be inferred that, after exerting a potent suppressive activity on a strongly reactive MLR, suppressor cells of the regulatory population inhibited the reactivity of the cells from their own spleen cell population, leading to an 'autoextinction' of reactivity of the splenocytes from allogeneically pregnant mice through recruitment and/or amplification of suppressor cells. For an efficient primary MLR stimulation, reactive cells do not need the continuous presence of stimulatory cells for more than 24 h. Since the MLR had been going on for 2 days already, a mechanism of suppression by cytotoxicity on stimulatory cells, as the one demonstrated by Fitch, Engers, Cerotinni & Brunner (1976) is highly unlikely to be the one accounting for the decreased reactivity of the control MLR.

Even more noteworthy is the fact that the regulatory cell population displayed an increased reactivity (as compared to one from virgin or isopregnant mice) on a 2 day duration MLR, similar (see kinetics in Chaouat & Voisin, 1979) to the kinetics of a secondary MLR. This is somehow reminiscent of the paradoxical effect described by Nadler & Hodes (1977) of allosensitized splenocytes displaying a heightened reactivity, leading nevertheless to a suppressive effect on MLR performed in 10% foetal calf serum. The significance of this phenomenon is still obscure: secondary MLR with suppressor population could act only on already triggered, differentiated lymphocytes. Ly 1^+, Ly $2,3^+$ feed back as recently suggested in antibody suppression by Eardley *et al.* (1978) could be a constant mechanism of immune regulation, and operate not only at the level of antibody production, but also in cell-mediated phenomena. Alternatively, the proliferating cell could be within the Ly 2, 3^+ population, as in the Con A system of Tse and Dutton and not have a suppressive effect *per se* (it could even be suppressed by suppressor T cells, and be a precursor of cytotoxic cells). Finally, it is difficult formally to rule out differential filler-feeder effects amongst allopregnant cells as opposed to control ones, but the effect of anti-Thy 1.2+C at optimal doses render this unlikely. It is also still possible that allopregnant cells exert their effects by amplifying the suppressor activity within the test MLR itself, in a similar fashion to the T_S1–T_S2 system suggested by Germain, Theze, Kapp & Benacerraf (1978), or Tada, Taniguchi & Tokuhisa (1977) in the regulation of antibody production. The corecognition requirement of the target cell by responder and regulator cell (to be published) together with the abolition of the suppressive effect by optimal doses of anti-Thy 1.2+C demonstrates that at least the first step is triggered within the population of cells from allopregnant mice itself.

The effect of the anti-serum at high dilution on the reactivity of cells from allopregnant mice, tested by a conventional MLR, suggests elimination of a suppressor cell population within the whole pool of splenocytes from allopregnant mice, a fact confirmed by the loss of their suppressive effect upon a control MLR. Passive enrichment in allo-reactive cells, themselves suppressive, can be ruled out by the fact that, both during calibration procedure and functional assays, cells are resuspended *in the same volume* as untreated controls (themselves at a concentration of $6{\cdot}5 \times 10^6$ cells/ml). Such a procedure, again, formally does *not* warrant fully against filler feeder effects, but these would then have to be differential according to the origin of the treated population. Thus we favour the deletion of a peculiar T-cell subset. Such a cell population could either have a high Thy 1.2 density on its surface (T_I?) or be more sensitive, merely, to the action of antiserum+C than other thymocytes. This would be in keeping with MLR suppressive effects of T_I cells described by Simpson & Cantor (1975), especially since we are here dealing with a microculture system. Finally, one should not rule out the possibility that the so-called Thy 1.2 antigen could, in fact, be composed of several specificities, which could be recognized differentially by dilution effect followed by *in vitro* assay. Such a possibility appears not completely theoretical after the description of different subsets of anti-T cell monoclonal antibodies recently demonstrated by L. A. Herzenberg (seminar at the 'Institut de Biologie Moléculaire', Paris, April 1979). Thus, the effect of anti-Thy 1.2+C at high dilution on the suppression could be either deletion of a peculiar T-cell subpopulation, or merely affecting the ratio 'immune' (e.g. proliferating) cells versus suppressor, a parameter already described as critical in the suppression of antibody synthesis (Doyle, Parks & Weigle, 1976).

Whichever the effect of the antiserum, it appears that, as in our previous system, (Chaouat & Voisin, 1979) the suppression is at least T-cell mediated and does not require the specific expression of the whole H-2 haplotype at the stimulator cell surface. The pre-

sent system shows that mitomycin-treatment of the regulator cells is not a prerequisite to their study. The treatment can be bypassed by playing with the time of introduction of these cells. This system also shows that regulator cells induced during allogenic pregnancy can act after the induction phase, at the time of the proliferative phase of a MLR. Whether the two systems (mitomycin-treated cells added on day 0 or untreated cells added on day 2) are concerned with the same suppressor population or two different populations will be considered in a further study.

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