

Inhibition of the mitogenic effects of plasma by a monoclonal antibody to somatomedin C

(insulin-like growth factor/BALB/c 3T3/DNA synthesis/cell cycle)

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ABSTRACT Immunoneutralization studies with a monoclonal antibody to somatomedin C (Sm-C) were undertaken to further determine the role of this peptide in cellular proliferation. For our model we used density-arrested cultures of BALB/c 3T3 cells. Transient exposure of these cells to platelet-derived growth factor enables them to respond to platelet-poor plasma by progressing through the G₁ stage and undergoing renewed DNA synthesis. In this system, the combination of nanogram concentrations of Sm-C and epidermal growth factor can fully substitute for plasma, and microgram concentrations of insulin can substitute for Sm-C by crossreacting with the Sm-C receptor. We now show that a monoclonal antibody to Sm-C, which in defined medium blocks the mitogenic effect of Sm-C but not insulin, also blocks the stimulation of DNA synthesis by human plasma or calf serum. Furthermore, by adding the antibody at progressively later times during G₁, we show that these cells escape from their dependence on Sm-C for DNA synthesis after traversing G₁ to a point at or near the G₁/S boundary.

The somatomedins are a family of polypeptide hormones that mediate many of the growth promoting actions of growth hormone (1-3). Somatomedin C (Sm-C) and insulin-like growth factor I (IGF-I) are synonyms for a single-chain 70-amino acid basic somatomedin that is more growth-hormone dependent and more mitogenic than the other human somatomedin, insulin-like growth factor II. The latter is a neutral molecule that is more insulin-like in its actions and is present at levels 3 times greater than those of Sm-C/IGF-I. Sm-C will restore linear growth in either hypophysectomized rats or in mice with a hereditary form of hypopituitarism (Snell dwarf strain) (4, 5). Although nanogram concentrations of Sm-C have been reported to promote DNA synthesis and/or cell replication in many different cell types, the most detailed studies have been made in mouse mesenchyme-derived BALB/c 3T3 cells (6). These cells, which become quiescent after having been grown to confluency in 10% serum, will respond to fresh serum-containing medium by progressing through the G₁ stage and undergoing renewed DNA synthesis. Platelet-poor plasma (PPP), which is prepared by removing formed blood elements before clotting takes place, will not stimulate these quiescent cells to traverse G₁ unless they are first exposed to platelet-derived growth factor (PDGF) (7). Stiles *et al.* (8) showed that PPP from hypophysectomized rats was ineffectual in stimulating DNA synthesis unless supplemented with added Sm-C. More recently, Leof *et al.* (9, 10) have shown that a combination of Sm-C and epidermal growth factor (EGF) can completely replace plasma in permitting PDGF-treated cells to progress to DNA synthesis. At high doses (1 μM), insulin can serve as a somatomedin surrogate by binding to the Sm-C receptor (11). The

development of a specific monoclonal antibody to Sm-C[§] has now made it possible to determine the consequences of selectively neutralizing this peptide in defined medium, in human plasma, and in calf serum.

MATERIALS AND METHODS

Cell Culture. BALB/c 3T3 cells (clone A-31) were cultured in a 1:1 mixture of Dulbecco's modified Eagle medium and Ham's F-12 (DME medium/F-12), supplemented with 10% calf serum. Cells were grown to confluence in 96-well microtiter plates (Nunc, Roskilde, Denmark) in 0.2 ml of serum-containing culture medium. Within 48 hr of reaching confluence, the medium was replaced for 5 hr with serum-free DME medium/F-12 containing PDGF at 20 ng/well. The PDGF-containing medium was replaced with culture medium containing [³H]methyl thymidine (6 Ci/mmol; 1 Ci = 37 GBq; Schwarz/Mann) at 1 μCi per well, and either plasma or growth factors with or without antibody. After 28 hr, the cells were fixed by the addition of 1 M ascorbic acid (0.06 ml per well), and the plates were processed for autoradiography or thymidine incorporation.

Thymidine incorporation was determined after the monolayers were rinsed in serum-free DME medium/F-12, fixed and washed twice in 5% trichloroacetic acid at 4°C and then solubilized in 0.1 M NaOH with 1% sodium dodecyl sulfate for counting. All results are expressed as the mean of triplicate wells ± SEM. In some experiments, autoradiography was performed according to the method of Antoniadis *et al.* (12).

Growth Factors and Sera. Sm-C was purified from human plasma by the method of Svoboda *et al.* (13). The preparation used in this study was a pool of side fractions from the terminal HPLC purification step. PDGF was prepared from heated extracts of clinically outdated human platelets chromatographed on CM Sephadex, followed by further purification on Blue Sepharose and Bio-Gel 150 as described (14, 15). EGF was prepared by the method of Savage and Cohen (16), and was the gift of E. O'Keefe. Human PPP (inactivated at 56°C for 30 min) was prepared as described by Pledger *et al.* (7) and calf serum was purchased from Hyclone (Logan, UT).

Monoclonal Antibody to Sm-C (sm-1.2). The monoclonal antibody to Sm-C was produced in a mouse hyperimmunized over 6 weeks with highly purified Sm-C conjugated to mouse albumin. Prior to fusion with P3X63Ag8.653 myeloma cells, the splenocytes were boosted *in vitro* by a 5-day reexposure to pure Sm-C in thymocyte-conditioned medium. Anti-soma-

Abbreviations: Sm-C, somatomedin C; PDGF, platelet-derived growth factor; PPP, platelet-poor plasma; EGF, epidermal growth factor; IGF-I and -II, insulin-like growth factors I and II.

[§]Van Wyk, J. J., Underwood, L. E., Gillespie, G. Y., Svoboda, M. E. & Russell, W. E. "Affinity Purification of Somatomedin-C and Immunoneutralization of Its Mitogenic Effects by a Monoclonal Antibody," Program of the Sixty-Fifth Annual Meeting of the Endocrine Society, June 8-10, 1983, San Antonio, TX.

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tomedin-producing hybrids were cloned and amplified by intraperitoneal injection into pristane-primed BALB/c mice. The immunoglobulin fraction from the clone designated sm-1.2 was further purified from ascites fluid by sequential precipitation in 18% and 15% sodium sulfate (17).

The antibody was a κ IgG₁ and bound Sm-C with an affinity constant of 1.09×10^{10} liters/mol. IGF-II (gift of R. Humbel, Zurich) and multiplication-stimulating activity (Collaborative Research, Lexington, MA) were, respectively, 5% and 1% as potent as Sm-C. The affinity of the antibody was negligible for chymotryptic fragments of Sm-C, various insulins, bovine proinsulin, human growth hormone, and EGF.

RESULTS AND DISCUSSION

Fig. 1A shows that PDGF-treated cells subsequently exposed to EGF and Sm-C or to 5% plasma exhibited high rates of [³H]thymidine incorporation into trichloroacetic acid precipitable material compared to PDGF-treated cells left in 0.1% PPP without exogenous growth factors. Whereas the monoclonal antibody, designated sm-1.2, had little influence on the baseline [³H]thymidine incorporation observed in 0.1% PPP, it sharply inhibited [³H]thymidine incorporation induced by either 5% PPP or by Sm-C and EGF. The residual stimulation of thymidine incorporation in the presence of plasma was not due to failure to inhibit IGF-II or other somatomedin-like peptides in plasma since the residual was identical in plasma-free medium containing only Sm-C and EGF. When nuclear labeling was used as an index of DNA synthesis (Fig. 1B), sm-1.2 produced a comparable inhibition of Sm-C-dependent labeling as that depicted in Fig. 1A. However, the antibody failed to influence the labeling observed when 1 μ g of insulin per ml was used as a somatomedin surrogate. This is in keeping with the previously documented high specificity of the antibody for Sm-C.

These results (Fig. 1) were identical whether soluble antibody was incorporated directly into the incubation medium or whether the experimental media were preincubated with Sepharose-bound sm-1.2 to remove the growth factor prior to exposure of the cells. Thus, the affinity of the antibody for Sm-C appears to be sufficiently high that the growth factor need not be physically removed from the solution to be rendered inactive.

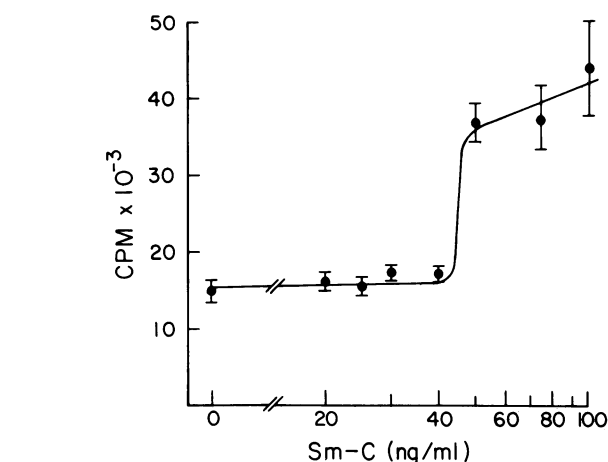
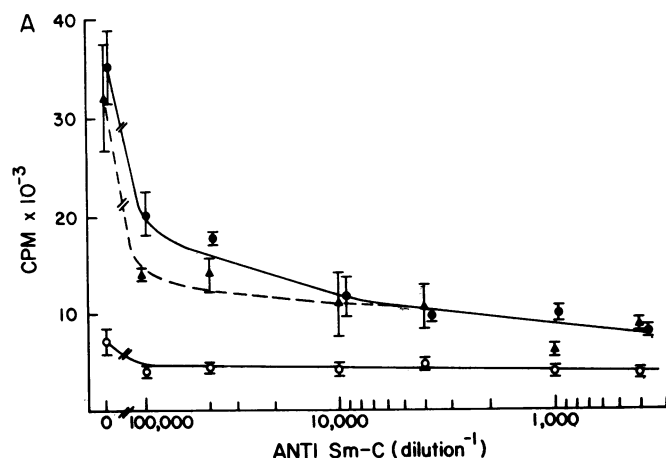


FIG. 2. The effects of excess Sm-C on the inhibitory effect of monoclonal antibody. Thymidine incorporation was determined as described in the text. After exposure to PDGF for 5 hr, increasing amounts of Sm-C were added to cultures containing EGF (10 ng/ml) and sm-1.2 (1:4000).

The inhibitory effect of the Sm-C antibody on DNA synthesis could be overcome by the addition of excess Sm-C. Fig. 2 shows that Sm-C at 40–50 ng/ml fully restored the mitogenic activity of culture medium containing EGF at 10 ng/ml and sm-1.2 at 1 μ g/ml. To exclude the possibility that the inhibitory effect of the monoclonal antibody might be due to some other factor in the ascites fluid and not to the antibody, we tested the effect of a similarly processed IgG monoclonal to horse apoferritin and observed no inhibition of the stimulatory effect of Sm-C and EGF or of PPP (data not shown).

The antibody was as effective in inhibiting the activity of 5% human plasma as it was against the combination of Sm-C and EGF. Complete inhibition of the mitogenic effect of human plasma was evident up to a plasma concentration of 10% (Fig. 3A). The antibody also decreased the potency of calf serum (Fig. 3B), but this effect was less complete. We attribute these differences between human plasma and calf serum to the presence of PDGF in calf serum since the re-

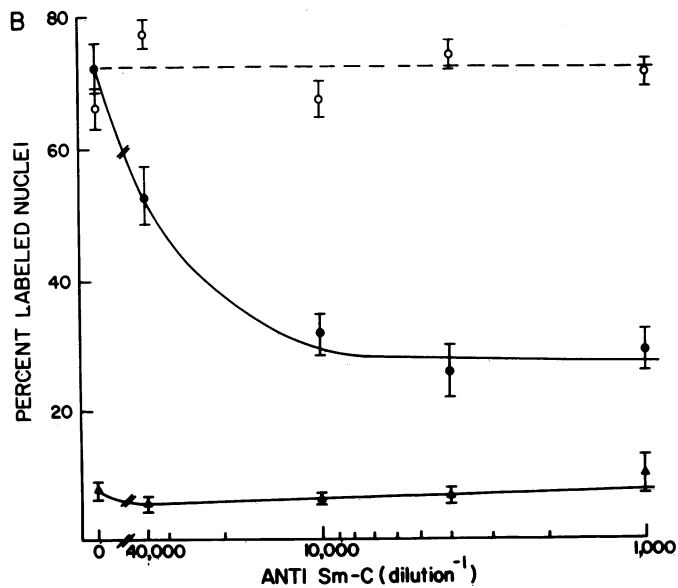


FIG. 1. The effects of monoclonal antibody on DNA synthesis induced in PDGF-primed BALB/c 3T3 cells by plasma and by the combination of EGF with Sm-C or insulin. (A) Thymidine incorporation. 0.1% PPP (○); Sm-C at 20 ng/ml and EGF at 10 ng/ml (●); and 5% PPP (▲). All results are expressed as the mean of triplicate wells \pm SEM. (B) Autoradiography. 0.1% PPP (▲); Sm-C at 20 ng/ml and EGF at 10 ng/ml (●); insulin at 1 μ g/ml and EGF at 10 ng/ml (○). Two predetermined fields were counted from each of two to four identical wells; results are expressed as percent labeled nuclei \pm SEM.

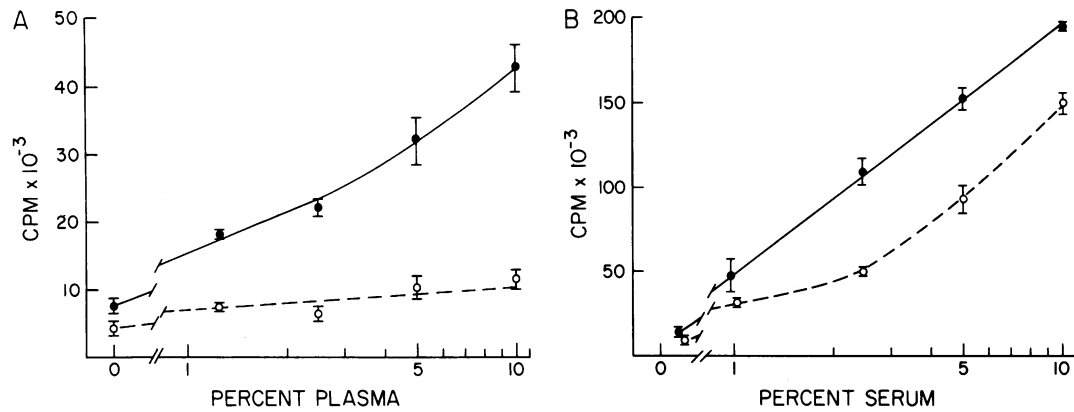


FIG. 3. The effects of monoclonal antibody (1:4000) on the DNA synthesis induced by human PPP (A) and calf serum (B). Thymidine incorporation was determined in the presence of plasma or serum alone (●) or in the presence of sm-1.2 diluted 1:4000 (○).

quirement of these cells for plasma (or Sm-C) is progressively decreased with increasing concentrations of PDGF (7). These data lend strong support to the hypothesis that Sm-C is a necessary component of plasma for the promotion of growth in cultured cells.

A consistent finding in our studies was that sm-1.2 produced a small inhibition of the DNA synthesis induced by EGF alone (Table 1). Since Leof *et al.* (10) found that EGF could not support the progression of BALB/c 3T3 cells through the early phases of G₁ unless low concentrations of Sm-C were simultaneously present, and since Sm-C is produced by many cell types *in vitro* (18), we postulated that the small inhibition of DNA synthesis caused by the antibody when only EGF was added to the medium was due to an inhibitory effect of the antibody on small amounts of endogenous Sm-C produced by the cells themselves. This was confirmed by finding that low but significant concentrations of immunoreactive Sm-C accumulate in the medium of density-inhibited BALB/c 3T3 cells cultured in the presence of PDGF and EGF (data not shown). This interpretation is further supported by the failure of the antibody to block the mitogenic effect of EGF in the presence of insulin when added in sufficiently high concentrations to serve as a Sm-C surrogate (Fig. 1B and Table 1).

To determine whether at some point in G₁ commitment to DNA synthesis no longer requires the presence of Sm-C, we added the monoclonal antibody at progressively later time intervals to the medium of PDGF-primed cells containing a combination of EGF and Sm-C and measured the rate of thymidine incorporation over 28 hr as described in Fig. 1A. As shown in Fig. 4, addition of the antibody at any time prior to hr 9 after adding Sm-C and EGF completely prevented entry into DNA synthesis; thereafter, an increasing percentage of the cells entered the S phase. Since the minimum transit time through G₁ after the addition of EGF and Sm-C is 10 hr in the absence of plasma (9), it can be concluded that irrevocable

commitment to DNA synthesis requires Sm-C and occurs approximately at the G₁/S boundary. These findings agree well with the "W" point of irrevocable commitment described by Pledger *et al.* (19).

The present studies were confined to the effect of the antibody to Sm-C on DNA synthesis, although in other studies (with Samuel D. Balk and David R. Clemmons) we have shown that this antibody also inhibits Sm-C-dependent cell proliferation in chicken heart mesenchymal cells and in porcine aortic smooth muscle cells, respectively. The studies reported here demonstrate the potential utility of a monoclonal antibody to Sm-C in defining the role of this peptide in cellular growth and in probing the mechanisms of its action at the cellular level. Our observation that confluent BALB/c 3T3 cells synthesize small amounts of immunoreactive Sm-C extends the observations of others (20–22) that many cell types are capable of somatomedin synthesis *in vitro*. Immunoneutralization appears to be a very potent tool for elucidating the role of somatomedin produced endogenously by cultured cells and organs. This "subtractive" approach will also help to define the contribution of Sm-C to the biologic effects of complex mixtures such as plasma and serum, although at the concentrations used in these studies the antibody might not unequivocally discriminate between Sm-C and IGF-II. Furthermore, this demonstration that the monoclonal antibody inhibits the mitogenicity of serum opens the

Table 1. Inhibition of mitogenic effects by sm-1.2 in the absence of added Sm-C

Conditions	Antibody sm-1.2 (1:4000)	cpm ± SEM
0.1% PPP	–	33,609 ± 868
	+	22,270 ± 1087
Insulin (1 μg/ml)	–	61,119 ± 2728
	+	61,291 ± 2295
EGF (10 ng/ml)	–	82,476 ± 1724
	+	57,195 ± 2097
EGF (10 ng/ml) and insulin (1 μg/ml)	–	126,364 ± 4494
	+	136,661 ± 2000

–, Without sm-1.2; +, with sm-1.2.

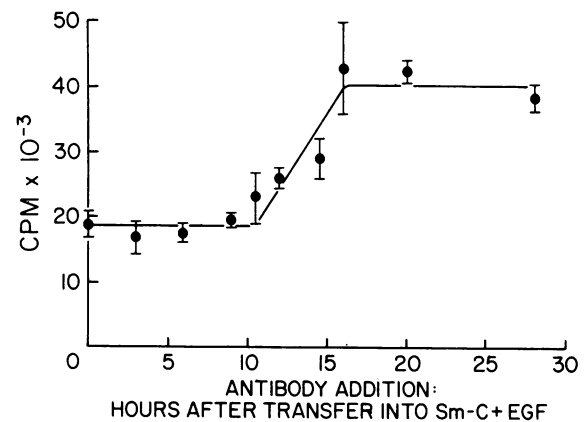


FIG. 4. The time course of Sm-C dependent commitment to DNA synthesis. PDGF-primed cells were exposed to medium containing Sm-C at 20 ng/ml, EGF at 10 ng/ml, and [³H]thymidine as described. At the times indicated after the addition of growth factors, the medium of triplicate wells was replaced with identical medium containing sm-1.2 diluted 1:4000. After 28 hr, the incorporation of [³H]thymidine was arrested by the addition of ascorbic acid and the cells were harvested.

way for its use to determine the biological role of the somatomedins in the growth of living animals.

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