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Role of the p53 Protein in Cell Proliferation as Studied by Microinjection of Monoclonal Antibodies

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Two monoclonal antibodies against the p53 protein, PAb 122 and 200-47, were microinjected into mammalian cells as a probe to determine the role of the p53 protein in cell proliferation. PAb 122 recognizes the p53 proteins of mouse and human cells but not of hamster cells, whereas 200-47 recognizes the p53 proteins of mouse and hamster cells but not of human cells. The ability of these antibodies to inhibit serum-stimulated DNA synthesis of cells in culture correlates with their ability to recognize the species-specific antigenic determinants. More important, however, is the observation that microinjected PAb 122 inhibits the transition of Swiss 3T3 cells from G_0 to S phase, but has no effect on the progression of these cells from mitosis to the S phase.

The p53 protein is a transformation-related protein that is often present in higher amounts in transformed cells than in their normal, untransformed counterparts (7, 10, 18, 25). It has been detected in cells transformed by DNA viruses (16, 17), by RNA viruses (25, 27), by chemicals and X-rays (10), and in several human tumor cell lines (7). Although present in uninfected embryonal carcinoma cells (17), it is not detectable in 3T3 cells (10) and in several untransformed cell strains (7). The synthesis of the p53 protein is markedly increased in mixed populations of lymphocytes stimulated by concanavalin A (21, 22). Its relationship to actively dividing cells has suggested to a number of investigators that the p53 protein may play a role in the regulation of cell proliferation (6, 11, 17, 22).

In a previous paper (19) we have shown that microinjection of a monoclonal antibody against the p53 protein (αp53) inhibited the entry into S phase of quiescent Swiss 3T3 cells stimulated by serum. The inhibitory effect was observed only when αp53 was microinjected about the time of serum stimulation. In this paper, we extended our studies on the effect of microinjected αp53 on cells from different species, on the accumulation of cellular DNA, and on the progression of Swiss 3T3 cells from mitosis through G_1 to S phase.

MATERIALS AND METHODS

Cell lines and culture conditions. The Syrian hamster G_1-specific, temperature-sensitive mutant cell line ts13 was maintained under culture conditions as previously described in detail (2, 12). The culture conditions for Swiss 3T3 mouse cells have also been described (19). The simian virus 40 (SV40)-transformed cell lines HR8 (TK^-ts13 Syrian hamster) and SV3T3 (Swiss 3T3 mouse) were established in our laboratory by transfection (28) and were routinely grown in Dulbecco modified Eagle medium with antibiotics containing 2% calf serum for HR8 or 2% fetal calf serum for SV3T3. The human cell strain WI-38 and an SV40-transformed cell line VA13-2RA derived from WI-38 were both kindly provided by V. J. Cristofalo (Wistar Institute). They were grown in Eagle minimum essential medium with antibiotics containing 15% fetal calf serum or 2% fetal calf serum, respectively.

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Quiescent cultures of nontransformed parental cells were established for microinjection by plating 2×10^5 to 4×10^5 cells per 60-mm petri dish in growth medium containing 1% calf serum, followed by 5 to 7 days of incubation at 37°C for Swiss 3T3 and WI-38 or at 34°C for ts13. At this time, the cells were quiescent (see below) and could be used for serum stimulation and microinjection.

Microinjection procedure. The glass-capillary microinjection method of Graessmann and Graessmann (14) and the modifications for antibody microinjection have been described previously (12, 19, 20). Briefly, a small circle was etched on a 22-mm^2 glass cover slip before the cells were plated for quiescence. All, or nearly all, of the cells within the circle were microinjected, and the cells outside of the circle (treated in exactly the same way except for microinjection) served as background controls. The efficiency of antibody delivery was determined by immunofluorescence after microinjection by staining with fluorescein-conjugated goat anti-mouse (or anti-rabbit) immunoglobulin G (IgG) as previously described and is fairly constant in various experiments (19, 20; W. E. Mercer, C. Avignolo, N. Galanti, K. M. Rose, J. K. Hyland, S. T. Jacob, and R. Baserga, Exp. Cell. Res., in press).

The IgG preparations for microinjection were obtained by ammonium sulfate precipitation of hybridoma culture medium, either from clone 122 (15) or from clone 200-47 (11). IgG prepared from Lyt 2.2 (19) or IgG from a nonimmunized rabbit (Mercer et al., in press) served as a control for microinjection.

Acridine orange staining and fluorescence measurements. Staining of DNA by acridine orange was carried out as follows. Swiss 3T3 cells were grown on cover slips and microinjected as described above. At different times after microinjection, the cells were first washed three times in Hanks balanced salt solution and then fixed in methanol-acetic acid (3:1) for 5 min at room temperature. The cover slips were soaked in Walpole acetate buffer (pH 4.2) (200 ml of 1 N sodium acetate and 140 ml of 1 N HCl) for 5 min and then stained for 3 min in the same buffer containing 0.05 mg of acridine orange (Polysciences Inc., Warrington, Pa.) per ml. After staining, the cover slips were washed for 5 min in buffer, rinsed in distilled water, air dried, and mounted on microscope slides. The fluorescence intensity of individual

cells (microinjected or nonmicroinjected) was measured with a computer-controlled microspectrofluorimeter with epi-illumination (Zeiss, Zonax). The amount of DNA was determined by measuring green fluorescence (F 520 to 560).

Autoradiography. Cultures were labeled with [^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) at 0.2 μCi/ml for continuous labeling experiments. When pulse-labeling was used, the cultures were labeled for 30 min with 20 μCi of [^{3}H]thymidine per ml. In experiments where the amount of DNA was determined by microspectrofluorimetry after acridine orange staining, the fluorescence intensity was measured before autoradiography, which was then carried out by standard procedures.

Immunoprecipitation and gel electrophoresis. Cells growing exponentially in 60-mm petri dishes (~10^6) were labeled for 6 h with [^{35}S]methionine (1,026 Ci/mmol; New England Nuclear Corp.) in methionine-free medium at 100 μCi per dish. At the end of the labeling period, Nonidet P-40 extracts were prepared and immune precipitated as previously described in detail (20). The immunoprecipitates were analyzed on 10% polyacrylamide-sodium dodecyl sulfate gels, followed by autoradiography.

Preparation and collection of mitotic cells. Mitotic cells were collected by hypotonic shake-off from exponentially growing cultures of Swiss 3T3 cells as described by Gaffney and McElwain (13). Under these conditions, the mitotic index at plating was consistently >90%.

RESULTS

In a previous report (19), we investigated the effect of monoclonal antibody 200-47 (11) microinjected into Swiss 3T3 cells. We extended these experiments to another monoclonal antibody against the p53 protein PAb 122 (15) and two other cell lines, ts13 and WI-38.

Inhibitory effect of p53 is dependent on antibody used. PAb 122 recognizes both mouse and human p53 (15), whereas 200-47 recognizes mouse but not human p53. We confirmed that PAb 122 immunoprecipitates a p53 protein from SV40-transformed mouse SV3T3 cells (Fig. 1, lane d) and a p53 doublet from human VA13-2RA cells (Fig. 2, lane d). The immunoprecipitation of a p53 doublet from SV40-transformed human cells with PAb 122 has also been reported by Crawford and co-workers (7). Different results were obtained with 200-47. This monoclonal antibody immunoprecipitates a p53 protein from SV40-transformed mouse cells (Fig. 1, lane c) and to a lesser degree from hamster HR8 cells (Fig. 3, lane c). The p53 band, immunoprecipitated from mouse and hamster cells by 200-47, migrates with the same mobility as the p53 band which was coprecipitated from both species with anti-T IgG (Fig. 1, lane a, and Fig. 3, lane a, respectively) but fails to precipitate a similar protein (p53 doublet) from SV40-transformed human cells. No p53 protein is detectable at this exposure in the immunoprecipitates of Swiss 3T3 and WI-38 cells, regardless of the antibody used, PAb 122, 200-47, or polyclonal anti-T. We have on occasion observed a faint band in TK$^-$ts13 cells, the parent cell line of HR8. Rotter and co-workers (26) have also found that PAb 122 recognizes mouse and human p53 but not hamster p53. Incidentally, the ability of monoclonal antibody 200-47 to immunoprecipitate hamster p53 has also been observed by this group. At long exposures, the hamster p53 band is very diffuse; however, with shorter exposures, a p53 triplet band can be resolved (V. Rotter, personal communication). The technique of indirect immunofluorescence also supports the finding of species-specific antigenic determinants of p53 (data not shown).

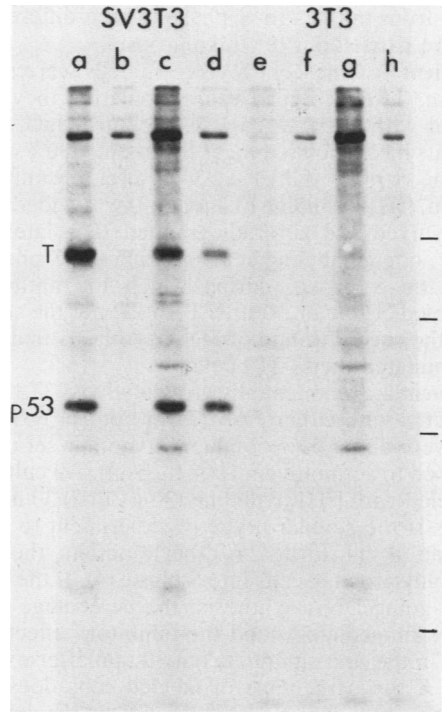

FIG. 1. Immunoprecipitation of [^{35}S]methionine-labeled proteins from mouse cell lines. Labeled proteins from SV3T3 (SV40 transformed) and 3T3 cells were immunoprecipitated with hamster antitumor IgG (anti-T) (lanes a and e); normal hamster IgG (lanes b and f); 200-47 IgG (lanes c and g); and PAb IgG (lanes d and h). The immunoprecipitates were electrophoresed and autoradiographed as described in the text. Molecular weight markers are indicated by horizontal lines and are, from top to bottom, phosphorylase B (92,500), bovine serum albumin (68,000), ovalbumin (43,000), and β-lactoglobulin (18,400).

We microinjected these two monoclonal antibodies into quiescent mouse 3T3, hamster ts13, and human WI-38 cells. Immediately after microinjection, the cultures were stimulated with 10% fetal calf serum. An unrelated monoclonal antibody, Lyt 2.2, known not to interfere with serum-stimulated cell DNA synthesis (19), was also microinjected as a control. A second control was provided by the nonmicroinjected background cells on the same cover slips but outside the circles of microinjected cells. The results are shown in Table 1. In no case did Lyt 2.2 inhibit serum-stimulated DNA synthesis, regardless of the cell line used. PAb 122, when microinjected at the time of serum stimulation, inhibited entry into S phase of Swiss 3T3 and, to a lesser extent, of WI-38 cells, but it had no effect on ts13 cells. On the contrary, 200-47 was inhibitory for ts13 and 3T3 cells, but it had no effect on WI-38 cells. It seems, therefore, that the inhibitory effect on DNA synthesis of the two monoclonal antibodies is correlated with their ability to recognize the species-specific antigenic determinants.

Microinjected PAb 122 does not inhibit the transition of cells from mitosis to S phase. Our previous results (19) and the present data indicate that microinjected PAb 122 (or 200-47) inhibits the serum-stimulated transition from G_0 to S phase. Since αp53 seems to be effective only when microinjected within 2 h after serum stimulation, we thought it would be worthwhile to investigate whether PAb 122 could inhibit the

transition from mitosis to S phase. Three different experiments were carried out for this purpose.

Experiment 1. Quiescent Swiss 3T3 cells were stimulated with serum. Twenty hours after stimulation they were microinjected with either PAb 122 or control IgG. (Previous experiments have shown that, at this time, >80% of the cells were in the S phase [19].) At 30 h after the initial serum stimulation, [^{3}H]thymidine (0.2 μCi/ml) was added. The cells were then fixed and autoradiographed 18 h later. By this procedure, one is labeling cells entering the S phase subsequent to the S phase, during which the antibodies are microinjected (Table 2). Neither PAb 122 nor the control IgG inhibited the second round of DNA synthesis that occurs in serum-stimulated Swiss 3T3 cells.

Experiment 2. Exponentially growing Swiss 3T3 cells were microinjected with either PAb 122 or control IgG when the cultures were about 50% confluent. Another set of cultures was exposed to actinomycin D (0.1 μg/ml). All cultures were pulse-labeled with [^{3}H]thymidine (20 μCi/ml) 30 min or 10 h later. This is the standard type of experiment to determine whether an agent (drug or other) inhibits the entry of exponentially growing cells into S phase (4). If the procedure affects on-going DNA synthesis, the percentage of labeled cells drops immediately, and the inhibitory effect is apparent, even in the first sample taken 30 min after exposure to the drug. If the percentage of labeled cells does not drop immediately, but does so at later times, the inhibition is indicative of a block in the flow of cells from previous stages into the S phase (Table 3).

Neither PAb 122 nor actinomycin D inhibits DNA synthesis when the cells are tested 30 min after microinjection or exposure to the drug, indicating that on-going DNA synthesis is not affected by these procedures. Ten hours later, the percentage of labeled cells in the actinomycin D-treated cultures dropped by 33%, i.e., actinomycin D inhibited the entry of cells into S phase (4). Under the same experimental conditions, microinjection of PAb 122 or control IgG has no effect on the percentage of labeled cells, indicating that neither antibody affects the flow of cells into the S phase.

TABLE 1. Effect of microinjected antibodies against the p53 protein on serum-stimulated DNA synthesis[a]

Cell line	Antibody microinjected	% Labeled cells[b]	% Inhibition
Mouse 3T3	None	49	
	Lyt 2.2	49	None
	200-47	23	52
	PAb 122	9	82
Hamster ts13	None	59	
	Lyt 2.2	60	None
	200-47	31	48
	PAb 122	57	None
Human WI-38	None	34	
	Lyt 2.2	37	None
	200-47	39	None
	PAb 122	17	46

[a] All cultures were made quiescent as described in the text and then microinjected with one of the three antibodies listed above in the table. The cells were then promptly stimulated by serum, [^{3}H]thymidine (0.2 μCi/ml) was added, and all cultures were fixed 17 h after serum stimulation.

[b] The percentage of labeled cells was determined by autoradiography. Nonmicroinjected cells are cells on the same cover slips outside the circles of microinjected cells.

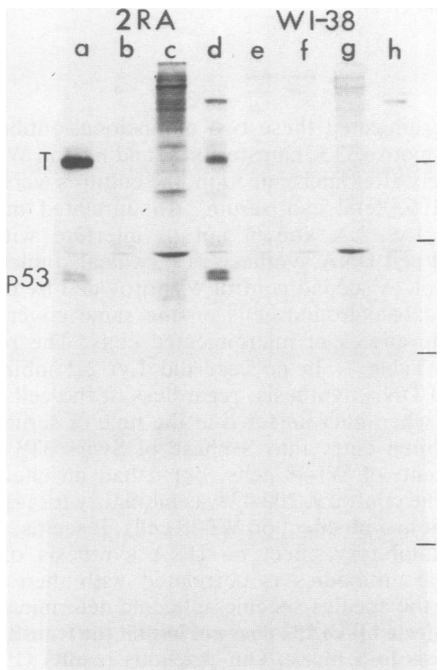

FIG. 2. Immunoprecipitation of [^{35}S]methionine-labeled proteins from human cell lines. The cell lines used were WI-38 human diploid fibroblasts and 2RA (SV40-transformed human fibroblasts). Antibodies, lanes, and other conditions are as described in the legend to Fig. 1.

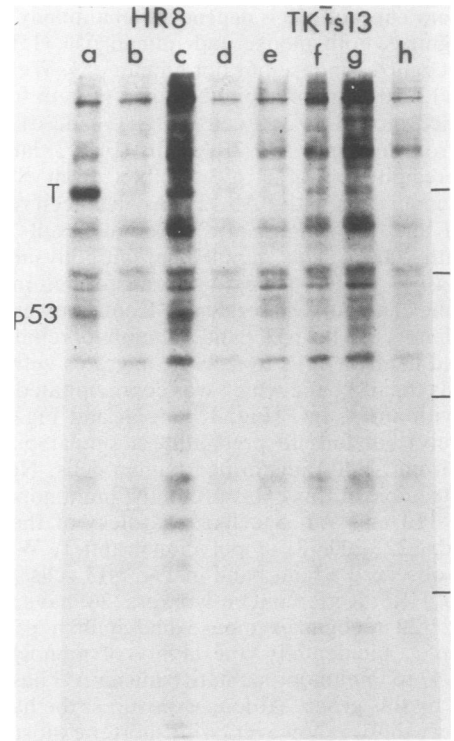

FIG. 3. Immunoprecipitation of [^{35}S]methionine-labeled proteins from hamster cell lines. HR8 are SV40-transformed TK$^-$ts13, which are in turn derived from Syrian BHK cells. Antibodies, lanes, and other conditions are as described in the legend to Fig. 1.

TABLE 2. Effect of microinjected antibodies on a second round of DNA synthesis

Antibody microinjected[a]	% Labeled cells
PAb 122	52
Control IgG	52
None	50

[a] Swiss 3T3 cells in the S phase (see text) were microinjected with either PAb 122 or control IgG. Ten hours later, when most of the cells had completed mitosis, [^{3}H]thymidine (0.2 μCi/ml) was added. The cells were labeled for an additional 18 h and then fixed and autoradiographed.

Since the G_1 phase of Swiss 3T3 cells, under these conditions, is about 8 h and the cells were followed for 10 h after microinjection, these results indicate that the microinjected antibodies have no effect on the progression of cells through G_1 phase.

Experiment 3. Mitotic Swiss 3T3 cells were collected from cultures of exponentially growing cells as described above. No drugs were used to arrest cells in mitosis. The mitotic cells (>90% pure) were plated, and within 1.5 h after replating they were microinjected with PAb 122 or control IgG; the nonmicroinjected cells served as a second control. [^{3}H]thymidine (0.2 μCi/ml) was added at the time of microinjection. The cells were fixed 10 h later. Figure 4 shows the histogram of the amounts of DNA for the three cell populations. They are indistinguishable from each other. This was confirmed by autoradiography. The percentage of labeled cells was 45 in control nonmicroinjected cells, 43 in cells microinjected with PAb 122, and 40 in cells microinjected with control IgG.

These three experiments demonstrate that microinjected PAb 122 does not affect the flow of Swiss 3T3 cells through G_1 phase.

Effect of PAb 122 on amount of cellular DNA in Swiss 3T3 cells. In these experiments, quiescent Swiss 3T3 cells were microinjected with either PAb 122 or Lyt 2.2 and then serum stimulated. The experiments were terminated 17 h later. The amounts of DNA in single cells were determined by computer-operated microspectrofluorimetry on fixed, acridine orange-stained cells. Quiescent, unstimulated cells and the stimulated cells outside the microinjection circle served as the negative and positive controls, respectively. Figure 5 shows the results for DNA. The amount of DNA per cell averaged 38 arbitrary units (a.u.) in unstimulated cells (Fig. 5a) and increased to 63 a.u. in serum-stimulated cells (Fig. 5b), as expected, since at 17 h, about 50 to 60% of the S phase was completed. In stimulated cells microinjected with Lyt 2.2, the mean DNA amount was 57 a.u. (Fig. 5c), which is not significantly different from that seen in Fig. 5b, but the amount in cells microinjected with PAb 122 was 38 a.u. (Fig. 5d). In this experiment, by autoradiography, the percent inhibition was 90%.

TABLE 3. Effect of microinjected antibodies on exponentially growing Swiss 3T3 cells[a]

Treatment	Time of labeling (h after treatment)	% Labeled cells
None[b]	0.5	49
PAb 122[b]	0.5	65
Control IgG[b]	0.5	60
Actinomycin D[b]	0.5	51
None[c]	10	49
Actinomycin D[c]	10	34
None[d]	12	40
PAb 122[d]	12	39
Control IgG[d]	12	35

[a] Exponentially growing Swiss 3T3 cells were microinjected with either PAb 122 or control IgG or incubated with actinomycin D (0.1 μg/ml). All cultures were pulse-labeled with [^{3}H]thymidine for 30 min and then fixed and autoradiographed. The variation in the percentage of labeled cells among control cultures reflects different experiments (in our methodology, the controls for microinjected cells are the nonmicroinjected cells outside the circles of microinjection).

[b] Cultures were pulse-labeled 30 min after treatment.

[c] Cultures were pulse-labeled 10 h after treatment.

[d] Cultures were pulse-labeled 12 h after treatment.

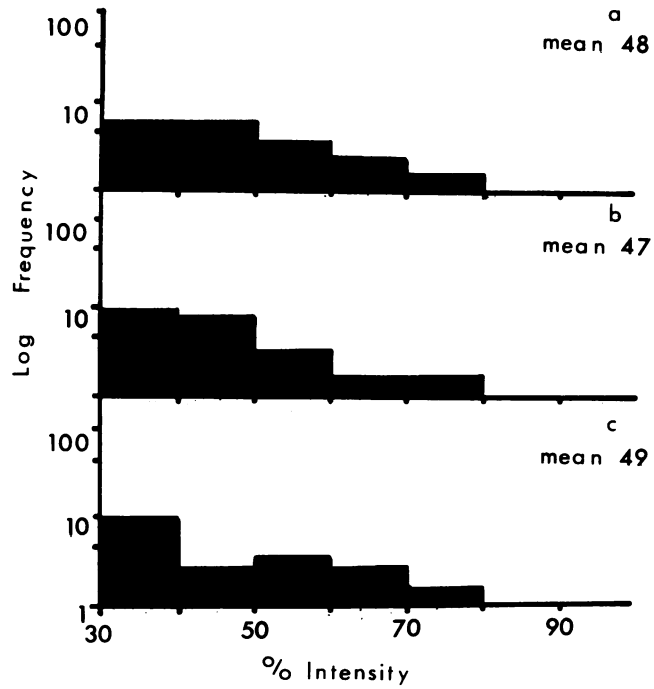

FIG. 4. Amount of DNA in Swiss 3T3 cells 10 h after mitosis. Cells collected by mitotic detachment were replated, and the amount of DNA in individual cells was determined 10 h later as described in the text. (a) Untreated cells; (b) cells microinjected with PAb 122; (c) cells microinjected with a control IgG. All cultures were in 10% fetal calf serum.

DISCUSSION

There is now convincing evidence in the literature that antibodies microinjected into mammalian cells preserve their specificity and are nontoxic so that they can be used to inhibit the function of the antigen against which they are directed. This statement is based on the following observations: (i) antibodies can be microinjected into cells without detectable toxicity (1, 12, 29); (ii) microinjected antibodies against SV40 T-antigen inhibit viral DNA replication (1) and SV40-induced cellular DNA synthesis (12, 20) but not serum-stimulated DNA synthesis (12); (iii) an antibody against RNA polymerase I inhibits, when microinjected, nucleolar RNA synthesis but not RNA synthesis directed by RNA polymerase II (Mercer et al., in press); (iv) control IgGs or control monoclonal antibodies microinjected into the nuclei of mammalian cells do not inhibit RNA synthesis (Mercer et al., in press) or serum-stimulated DNA synthesis (12, 19).

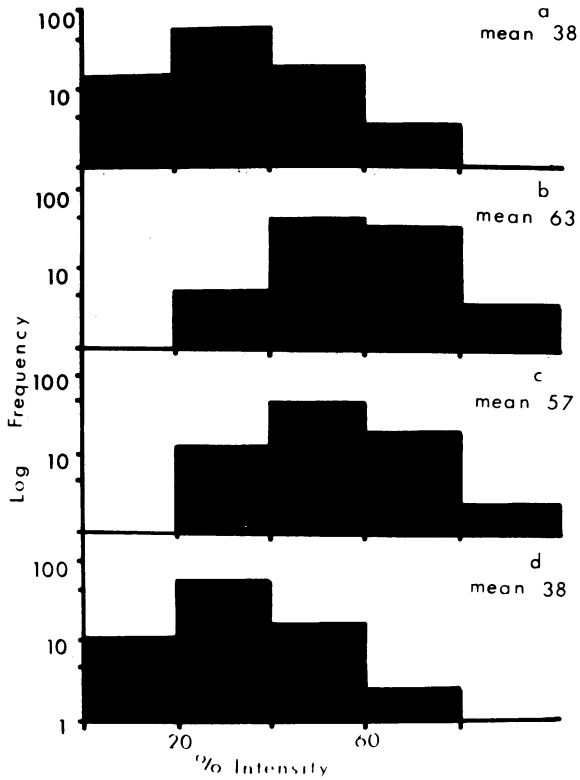

FIG. 5. DNA content of Swiss 3T3 cells. DNA content of individual cells was determined by measuring the green fluorescence of acridine orange-stained cells with a computer-operated microspectrofluorimeter. Staining intensity is expressed in a.u. (abscissa). (a) Quiescent, unstimulated cells; (b) cells stimulated with 10% serum for 17 h; (c) same as (b), but microinjected with Lyt 2.2; (d) same as (b), but microinjected with PAb 122.

The validity of the acridine orange procedure for the microspectrofluorimetric determination in single cells of the amounts of RNA and DNA is also well established in the literature (2, 8, 9). Green fluorescence is an accurate measurement of the amounts of DNA, whereas red fluorescence ($F > 590$) is a good indication of the amount of RNA, as 90% of the red fluorescence (and none of the green fluorescence) is eliminated by digestion with RNase.

The present experiments confirm that Lyt 2.2 (a monoclonal antibody of the same class as 200-47) and control IgG, when microinjected into serum-stimulated cells, have no effect on the entry of cells into S phase. Two novel findings brought forward by the data presented in this report are of particular interest. (i) The inhibitory effect of microinjected αp53 is correlated with the ability of the antibody to recognize species-specific antigenic determinants. (ii) By three different methods, microinjected αp53 does not inhibit the flow of cells through G_1 phase, whereas the same antibody almost completely inhibits serum-stimulated DNA synthesis when microinjected at the time of serum addition (Fig. 5). These findings deserve some further comments.

In a previous paper (19), the finding that αp53 inhibited serum-stimulated DNA synthesis only when microinjected at the time of stimulation (±2 h) suggests that the p53 protein may play a role in the exit of cells from the G_0 phase or quiescence, or resting stage, or whatever name one wishes to use for noncycling cells (3, 5, 23). This suggestion receives further support from the present finding that microinjected αp53 does not inhibit the flow of cells through G_1 phase. We have determined this by three different experiments. The first two (see above) were carried out on unperturbed cell populations; the third one was carried out on postmitotic cells 1.5 h after replating.

The postmitotic cells could not be injected earlier, because the cells must be allowed to spread before they can be microinjected. It could be argued that during that 1.5 h, the cells passed the block point determined by the microinjected αp53. However, if this were true, we should have observed an inhibition in the other two experiments.

There are at least two alternatives to explain the observation that microinjected αp53 inhibits the progression of cells from G_0 to S phase but not from mitosis to S phase: (i) p53 is not needed for G_1 progression; or (ii) cells in the G_1 phase have more p53 than do G_0 cells (22), and the amount of microinjected antibody is not sufficient to neutralize the increased amount (the constraints of the microinjection technique do not allow us to increase the amount of antibody delivered to the cell). In either case, the p53 protein seems to play a role, qualitative or quantitative, in the transition of Swiss 3T3 cells from a resting to a growing stage.

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