Table 1   Effect of nucleoside–IgG2a conjugate on appearance of anti-nucleoside antibody forming cells

Treatment 10 d before immunisation	Direct (AGCT) PFC per spleen on day 7	P	Indirect (AGCT) PFC per spleen on day 7	P
None	10,173±1,867		1,862±594	
AGCT–IgG2a	1,888±480	<0.001	498±202	<0.01

Seven BALB/c mice were treated with 0.2 mg of AGCT–IgG2a, given i.v. and, together with seven untreated mice, were immunised i.p. with 0.2 mg of AGCT–KLH in complete Freund's adjuvant 10 d later. Direct and indirect PFC were measured on day 7, as described in the legend to Fig. 1. Values are means ± s.e.

Table 2   Nucleoside specificity of tolerance

Treatment 10 d before immunisation		Direct PFC per spleen on day 6					
	AGCT–SRBC	P	A–SRBC	G–SRBC	P	C–SRBC	T–SRBC
None	19,970 (7,283)		1,055±922	8,848±3,538		6,706±3,730	7,943±4,546
G–IgG2a	12,751 (7,283)	>0.4	1,535±689	883±710	<0.01	5,824±4,791	6,067±2,066

Seven BALB/c mice were treated with 0.2 mg of G–IgG2a and, together with seven untreated mice, were immunised i.p. with 0.2 mg of AGCT–KLH in complete Freund's adjuvant 10 d later. Individual nucleoside–SRBC target cells were prepared as in the legend to Fig. 1, but in each case 10 mg of nucleoside were oxidised in 1.0 ml of 0.1M sodium periodate. Direct PFC were measured 6 d after immunisation. Values are means±s.e.

immunised with all four nucleosides on the immunogenic carrier, there was some variation in the number of PFC specific for each nucleoside. This reflected at the cellular level of the immunodominance of guanosine and the low immunogenicity of adenosine that we have noted previously for serum antibody responses[4]. Finally, from a technical point of view, the modifications of the technique of Erlanger and Beiser[5] to link nucleosides to sheep cells should be noted. The ionic strength had to be maintained to prevent lysis. The use of 0.15 M bicarbonate did this and provided a suitable pH for the Schiff base formation. The use of t-butylamine borane complex in place of sodium borohydride was important in preventing damage to the erythrocytes during reduction of the Schiff base, and the reduction time was brief.

The immune response to nucleic acid antigens is important from at least two points of view. First, since anti-nucleic acid antibodies are involved in autoimmune disease[8], the suppression of this response can be of therapeutic importance; and second, strain differences in the ability of mice to respond to nucleic acid antigens have been observed[9] and might offer the opportunity to study the genetic basis of both immunity and tolerance to defined determinants of naturally occurring antigens. The development of an assay that permits the detection of antibody forming cells to either all four nucleosides simultaneously or to individual nucleosides is useful for the study of these phenomena.

B. DAVID STOLLAR

Department of Biochemistry and Pharmacology,
Tufts University School of Medicine,
Boston, Massachusetts 02111

YVES BOREL

Division of Immunology,
The Children's Hospital Medical Center,
Department of Pediatrics,
Harvard Medical School,
Boston, Massachusetts 02115

1 Jerne, N. K., Nordin, A. A. & Henry, C. L. in *Cell Bound Antibodies* 109–116 (Wistar Institute, Philadelphia, 1963).
2 Cunningham, A. J. *Progr. Allergy* 17, 5–50 (1973).
3 Stollar, B. D. & Borel, Y. *J. Immun.* 115, 1095–1100 (1975).
4 Stollar, B. D. & Borel, Y. *J. Immun.* 117, 1308–1313 (1976).
5 Erlanger, B. F. & Beiser, S. M. *Proc. natn. Acad. Sci. U.S.A.* 52, 68–74 (1967).
6 Golan, D. T. & Borel, Y. *J. exp. Med.* 134, 1046–1061 (1971).
7 Borel, Y., Lewis, R. M. & Stollar, B. D. *Science* 182, 76–78 (1973).
8 Koffler, D. *J. exp. Med.* 126, 607–623 (1967).
9 Fuchs, S., Mozes, E. & Stollar, B. D. *J. Immun.* 114, 1287–1291 (1973).

T-cell cytotoxicity in the absence of viral protein synthesis in target cells

CYTOTOXIC T cells lyse only those virus infected target cells *in vitro* which express, in addition to the viral antigen(s), those *K* or *D* region products of the major histocompatibility complex (MHC) which were present during anti-viral sensitisation *in vivo*. This 'associative recogniton' by cytotoxic T cells could reflect the interaction of two T-cell receptors with specificity for target *K* or *D* gene products and independently for the viral antigen, or one receptor
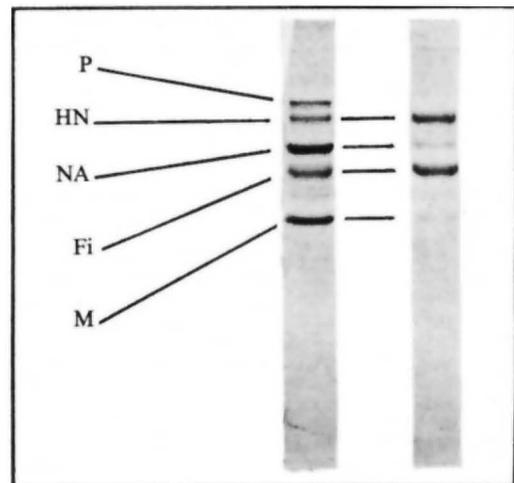
Fig. 1 Separation of Sendai virus envelope protein on SDS–polyacrylamide gels. Sendai virus (8 mg ml⁻¹) in phosphate-buffered saline without Mg or Ca (PBS-A), 1.5×10^5 haemagglutinating units (HAU) per ml were treated with 0.25% NP 40 for 15 min at 20 °C (ref. 6). The suspension was centrifuged at 120,000g for 1 h at 4 °C to remove partially disrupted virions and nucleocapsids. The supernatant was dialysed for 4 d at 4 °C against twice daily changes of PBS–A using spectrophor 2 tubing (Raven Scientific). The reassembled membrane particles were concentrated by centrifugation at 120,000g for 1 h at 4 °C and the pellet was resuspended in PBS–A. Envelopes were active in assays for haemagglutination[7], neuraminidase[8] and haemolytic activities[9]. Envelopes were analysed by SDS–polyacrylamide electrophoresis by the method of Laemmli[10] using 10% gels. P, polymerase; HNA, haemagglutinin; NA, Neuraminidase; F₁, fusion factor; M, M-protein.

with specificity for virally altered K or D region products (see ref. 1 and refs therein). There are various ways that the MHC antigens could be altered, including 'modification from within', where the virus modifies host protein synthesis by interfering with transcription[2], translation or post-translational glycosylation; or 'modification from without' where enzymic or chemical alteration of cell membrane proteins are induced by virus activity at the cell surface. In this report we show that inactivated Sendai virus or isolated Sendai virus envelopes can serve to modify a cell and make it a specific target for Sendai-immune T-cell killing, thus excluding the possibility of 'modification from within' in this system.

Sendai virus-primed BALB/c spleen cells, boosted *in vitro* with syngeneic infected blast cells, specifically killed Sendai virus-infected syngeneic target cells; primed spleen cells boosted with syngeneic non-infected cells did not (Table 1). Killer cells after secondary stimulation were T cells as shown by treatment with anti-Ly-sera (U. K. and P. Beverley, unpublished). Three approaches were used to show that synthesis of viral protein is not required in the target cell in order for it to be killed by virally sensitised T cells. First, cells incubated with ultraviolet-irradiated Sendai virus were lysed by sensitised T cells (data not shown). Second, cells exposed to purified Sendai virus, inactivated with β-propiolactone—which abolishes induction of protein synthesis by inactivated particles[5]—also proved to be effective target cells (Table 1). Treatment with β-propiolactone reduced the number of plaque forming units by 10^8. Since the ratio of particles to plaque forming units would be expected to be no better than 10 : 1 (J. Skehel, personal communication) and target-to-particle ratio was 1 : 10^6, it follows that, at most, one in a thousand cells were infected with a live virus. Sendai virus specific killer cells generated by secondary stimulation *in vitro* lyse only *H–2*-compatible infected target cells (U. K., unpublished) and the same restriction is seen using target cells incubated with inactivated Sendai virus (Table 2). Third, cells exposed to isolated envelopes from Sendai virus, which contained mainly the two surface glycoproteins with a very small amount of nucleocapsid and almost no detectable M-protein (Fig. 1), were susceptible to T cell-mediated lysis. To exclude nonspecific effects of the Sendai envelopes on target cells, influenza virus-specific cytotoxic T cells were tested on these target cells, and found to be ineffective (Table 3).

In several virus infections, target cell formation requires translation of early viral proteins[11-13], glycosylation[14], and
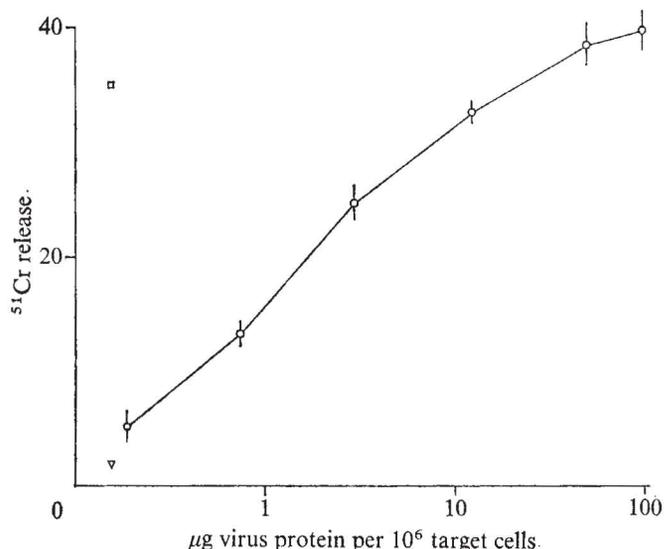
Fig. 2 T cell-mediated lysis of P-815 target cells exposed to dilutions of purified Sendai virus inactivated with β-propiolactone (B-PL). Sendai virus was grown in the allantoic cavity of 10-d-old embryonated fowl eggs and purified by centrifugation through a sucrose gradient (20–55% in PBS-A). The virus-containing band was collected and concentrated by centrifugation at 120,000g for 1 h. Inactivation was achieved by incubating the purified virus (2 mg per ml, 4×10^4 HAU per ml, 1 μg $\approx 10^{11}$ virus particles) for 18 h at 4 °C with β-propiolactone (Sigma) at a concentration of 0.025%. β-propiolactone was inactivated by incubation for 30 min at 37 °C. Inactivation was tested by egg infectivity titration[15]. ○—○, Lysis of P-815 cells exposed 2 h before test with dilutions of B-PL-Sendai virus (100 μg virus per 10^6 cells, about 10^5 particles per cell). ☐, P-815 targets infected overnight with 20 PFU per cell (1 PFU $\leqslant$ 10 particles). ▽, Non-infected P-815 control cells. Assay conditions identical to Table 1, incubation time 3 h, ratio of killer–target cells, 5:1.

expression of viral proteins on the cell surface. The ability of Sendai virus to by-pass these processes may be due to its unique capacity to mediate cell–cell fusion, which could allow the insertion of large amounts of virus glycoprotein into the target cell membrane. From the dose–response curve (Fig. 2) of target cell formation with dilutions of β-propiolactone inactivated Sendai virus, it can be seen that about 10^6 particles per target cell were required to induce

Table 1 Lysis of syngeneic target cells incubated with infective and inactivated Sendai virus or vesicles containing viral envelope proteins

Responder cells	Stimulator cells	Culture day	Ratio K:T	Targets			
				P-815	P-815/Sendai	P-815/B-PL-S	P-815/Sendai env.
BALB/c, Sendai immune	BALB/c con A treated blasts, Sendai infected	5	10:1	1.1	43.5	42.0	18.1
			5:1	0.3	26.6	26.3	9.7
			2.5:1	1.7	13.9	15.1	6.3
BALB/c, Sendai immune	BALB/c con A treated blasts	5	10:1	2.8	0.5	1.3	0.4
			5:1	3.5	0.9	0.9	1.0
BALB/c, Normal	BALB/c con A treated blasts, Sendai infected	5	10:1	0.1	0.9	4.0	1.6
			5:1	0.6	0.4	3.6	0.5

BALB/c mice were immunised to Sendai virus by i.p. injection of 0.2 ml allantoic fluid containing 10^7 plaque forming units (PFU) of virus[3]. Six weeks later spleens were collected and incubated in RPMI medium containing 10% foetal calf serum, glutamine and 5×10^{-5} M 2-mercaptoethanol in 2 ml well linbro plates in presence of stimulated cells. Stimulator cells were prepared by incubation of spleen cells in culture medium containing 2.5 μg ml^{-1} concanavalin A (con A) for 48 h followed by overnight infection with 20 PFU per cell Sendai virus and 2,000 rad irradiation. 5×10^6 responder cells were incubated with 5×10^5 stimulator cells. Cytotoxic T cells were collected from cultures 4–7 d later[4] and a ^{51}Cr release assay was performed with 5×10^3 syngeneic tumour target cells per well[3]. Target cells were *a*, ^{51}Cr labelled non-infected P-815 cells; *b*, P-815 cells infected overnight with 20 PFU per cell Sendai virus; *c*, P-815 cells pre-incubated for 2 h with β-propiolactone (B-PL) inactivated (see legend Fig. 2), purified Sendai virus in a protein concentration of 50 μg per 10^6 target cells; *d*, P-815 cells pre-incubated for 2 h with Sendai envelope (env.) proteins (see legend to Fig. 1) in a protein concentration of 50 μg per 10^6 target cells. Results are from triplicate assays; S.E.M. were always less than 3. Spontaneous lysis, which did not exceed 15–20%, is subtracted.

Table 2 Lysis of target cells treated with β-propiolactone inactivated Sendai virus is *H–2* restricted

Responder cells	Stimulator cells	P-815 S-B-PL ($H-2^{dd}$)	L-929 S-B-PL ($H-2^{kk}$)	EL-4 S-B-PL ($H-2^{bb}$)
BALB/c normal ($H-2^{dd}$)	BALB/c con A treated blast, Sendai infected	3.1	1.4	2.5
BALB/c immune ($H-2^{dd}$)	BALB/c con A treated blasts, Sendai infected	46.1	1.5	11.9
CBA immune ($H-2^{kk}$)	CBA con A treated blasts, Sendai infected	2.4	30.6	3.0
C57BL6 immune ($H-2^{bb}$)	C57 BL6 con A treated blast, Sendai infected	—	3.8	32.5

Conditions of immunisation and *in vitro* restimulation were as for Table 1. Cells were collected and tested on culture day 6. Target cells were incubated with B-PL-virus, in a protein concentration of 50 µg per 10^6 target cells. Results are from triplicate assays, ratio of killer–target cells is 20:1, incubation time 4 h.

a comparable degree of killing as seen with infected target cells. Since the percentage of particles which actually fuse with the target cells is unknown, the number of fused particles per cell necessary for target cell formation cannot be estimated. Failure to use sufficiently high concentrations of virus during target cell incubation may explain previous negative findings (ref. 12, and U.K., unpublished).

β-propiolactone-Sendai virus-treated target cells were lysed to a similar extent as target cells incubated with live virus, but target cells which were produced with Sendai virus envelope vesicles were consistently lysed at a lower plateau level. Two explanations are possible. There could be two populations of Sendai virus specific cytotoxic T cells

within'. If there are alterations of *H–2* gene products these must occur from without, at the cell surface. Envelopes contain neuraminidase activity, but cleavage of neuraminic acid seems not to change the *H–2* surface antigen specificity[16,17]. We do not know if there are other residual enzyme activities such as proteases or glycosidases remaining in the envelopes. It is also possible that cross-linking of sialic acids by the viral haemagglutinin might change the surface distribution of *H–2*, and sometimes create effective target antigens.

Which then are the viral antigens on cell surfaces recognised by the T cells? Recent studies on influenza virus-specific CTL have suggested that the M-protein which is

Table 3 Virus-specific lysis of target cells incubated with inactivated Sendai virus or Sendai virus envelopes

Responder cells	Stimulator cells	Culture day	Ratio K:T	Targets P-815	P-815/influenza	P815/Sendai	P-815/B-PL-S	P-815/Sendai env.
BALB/c, Sendai immune	P-815, Sendai infected	5	10:1	4.3	2.3	39.6	28.1	19.3
			5:1	−2.5	3.0	37.8	19.8	14.2
BALB/c, influenza immune	P-815, influenza infected	5	10:1	−0.9	27.4	0.8	1.3	1.8
			5:1	0.2	13.9	0.3	−3.8	3.9
(C3H×DBA/2) F_1, Sendai immune	BALB/c LPS blasts, Sendai infected	5	10:1	−0.29	3.7	26.5	27.2	—
			5:1	1.02	1.4	8.6	8.5	—
(C3H×DBA/2) F_1, influenza immune	BALB/c LPS blasts, influenza infected	5	10:1	0.3	19.8	3.4	3.8	—
			5:1	2.9	11.3	0.5	2.9	—

Responder cells (BALB/c, (C3H × DBA/2) F_1) were collected from mice injected i.p. 6 weeks previously with 10^7 PFU Sendai virus or influenza virus. The influenza strain A/Jap/Bel/305/57 (H_2N_1) a recombinant of A/Jap/305/57 (H_2N_2) and A/Bel/42 (H_0N_1) (kindly supplied by Dr J. Skehel) was used. Conditions of *in vivo* sensitisation and *in vitro* boosting of cells were similar to those used for secondary cytotoxic T-cell generation against Sendai virus. Stimulator cells were BALB/c lymphocyte blasts after 48 h incubation in 50 µg ml⁻¹ lipopolysaccharide containing medium or P-815 tumour cells. Stimulator cells were infected overnight with 20 PFU per cell and irradiated with 2,000 rad (blasts) or 8,000 rad (P-815). Ratio of stimulator to responder cells and ^{51}Cr release assay conditions were identical to those described in the legend to Table 1.

with receptors for different viral antigens; however, antigens on envelope-treated target cells are at least partially shared with those of infected target cells since the latter compete in cold cell inhibition tests with the lysis of target cells exposed to Sendai virus envelopes (data not shown). The second possibility is that the envelope vesicles are less active in fusing with the target cell membrane; this is the more likely explanation since the β-propiolactone inactivated virus was active in cell–cell fusion, while the envelopes were not.

These results rule out, at least for Sendai virus infection, formation of an 'altered self' by a 'modification from

not glycosylated and not expressed on cell membranes may contribute to target specificity[18]. This is very unlikely for Sendai virus since the envelope preparations contain only two glycoproteins, one with neuraminidase and haemagglutinin activity and the other with fusion activity.

These results show for the first time that target cells for virus-specific T-cell lysis can be prepared with vesicles containing essentially only viral surface glycoproteins and that viral protein synthesis is not required for target cell formation.

U. KOSZINOWSKI

ICRF Tumour Immunology Unit,
Department of Zoology,
University College London,
Gower Street, London WC1, UK

M. J. GETHING
M. WATERFIELD

Imperial Cancer Research Fund Laboratories,
Lincoln's Inn Fields,
London WC2, UK

1 Munro, A. & Bright, S. *Nature* 264, 145–152 (1976).
2 Garrido, F., Schirrmacher, V. & Festenstein, H. *Nature* 259, 228–229 (1976).
3 Ertl, H. & Koszinowski, U. *Immunobiology* 152, 128–140 (1976).
4 Gardner, I. D. & Blanden, R. V. *Cell Immun.* 22, 283–296 (1976).
5 Neff, J. & Enders, J. F. *Proc. Soc. exp. Biol. Med.* 127, 260–267 (1968).
6 Hosaka, Y. & Shimizu, Y. K. *Virology* 49, 627–639 (1972).
7 Salk, J. *J. Immun.* 49, 87–98 (1944).
8 Aminoff, D. *Biochem. J.* 81, 384–392 (1961).
9 Shimizu, Y. K., *J. Virol.* 9, 842–852 (1972).
10 Laemmli, U. K. *Nature* 227, 680–685 (1970).
11 Ueda, Y. & Tagaya, I. *J. exp. Med.* 138, 1033–1043 (1973).
12 Ada, G. I., Jackson, D. C., Blanden, R. V., Tha Hla, R. & Bowern N. A. *Scand. J. Immun.* 5, 23–30 (1976).
13 Koszinowski U. & Ertl, H. *Eur. J. Immun.* 6, 679–683 (1976).
14 Jackson, D. C., Ada, G. I., Hapel, A. J. & Dunlop, M. B. C. *Scand. J. Immun.* (in the press).
15 Scheid, A. & Choppin, P. W. *Virology* 62, 125–133 (1974).
16 Sandford, B. H. & Codington, J. F. *Tissue Antigens* 1, 153–161 (1971).
17 Ray, B. K. & Simmons, R. L. *Proc. Soc. exp. Biol. Med.* 142, 846–852 (1973).
18 Effros, R. B., Doherty, P. C., Gerhard, W. & Bennink, J. *J. exp. Med.* (in the press).

Cellular inactivation by ultrasound

THE lethal effect of ultrasound (US) on mammalian cells has received relatively little attention. Understandably, potential genetic aspects of US have been of prime concern to physicians who use US as a diagnostic tool; at the average power densities involved ($\ll 1$ W cm^{-2}) little, if any cell killing is to be expected. There have been sporadic attempts to use higher intensities (~ 1 W cm^{-2}) as a treatment modality in cancer therapy[1,2], but those experiments seem to have been based on inadequate cellular studies. The effects of US usually were evaluated in terms of morphological criteria rather than on quantitative determination of the loss of viability as measured by colony formation. There are few reports of the effects of US on survival of mammalian cells[3,4], and none specifically examine hyperthermic interaction. With the increased interest in hyperthermia for tumour therapy, attention has been directed towards the use of ultrasound to achieve tumour heating. In preliminary experiments in which US was used to heat the EMT6 sarcoma and KHJJ carcinoma in mice, we found a high percentage of tumour cures with short (~ 30 min) treatments at temperatures (43–44 °C) where *in vitro* results of hyperthermia-induced cell killing[5-7] would not have led to a prediction of any cures. We therefore initiated an investigation of the effects of US on survival of Chinese hamster cells to see if direct cell killing by US could explain our *in vivo* results, or, as in the case of radiofrequency (RF) electromagnetic heating, we would be forced to invoke host response[8]. In particular, we examined the thermal and non-thermal components of cellular inactivation by US. We report here that there is a definite non-thermal cytotoxic effect of US. Its relative contribution to cell killing is a highly nonlinear function of the temperature of the cellular milieu. The survival curves show clearly that, beyond an initial threshold, small changes in temperature and/or US intensity can give rise to impressive changes in survival values. The threshold nature of the data strongly suggests that by means of overlapping beams, ultrasound energy could be delivered to tumour tissue to achieve massive cell killings while

sparing normal tissue outside the tumour volume to a degree far exceeding that of conventional techniques.

Chinese hamster cells (HA-1) of ovarian origin were maintained in Eagle's MEM supplemented with 15% foetal calf serum, penicillin, and streptomycin. The cultures were kept in a humidified incubator with a mixture of 95% air and 5% CO$_2$, and were routinely checked for mycoplasma. To maximise the transmission of the acoustic energy and hence minimise heat induced by absorption of US, cells were grown as monolayers in specially designed stainless steel dishes with Mylar bottoms (130 μm thick). Approximately 8×10^4 cells were seeded within a confined area (1.6 cm in diameter) on the thin Mylar film; experiments were always performed on the third day after plating when the cell density reached approximately 5×10^5 cells cm^{-2} and the cells were still in exponential growth. Plating efficiencies of cells trypsinised from the Mylar film were 70–90%. Some experiments were performed with cells in suspension; as far as possible, the geometry was identical to that used for attached cells. The cloning technique of Puck and Marcus was used to determine the fraction of viable cells. The US irradiating system consisted of a laboratory-developed signal generator and power amplifier driving a piezo-electric transducer (Mettler Electronics). The transducer was mounted on the frame
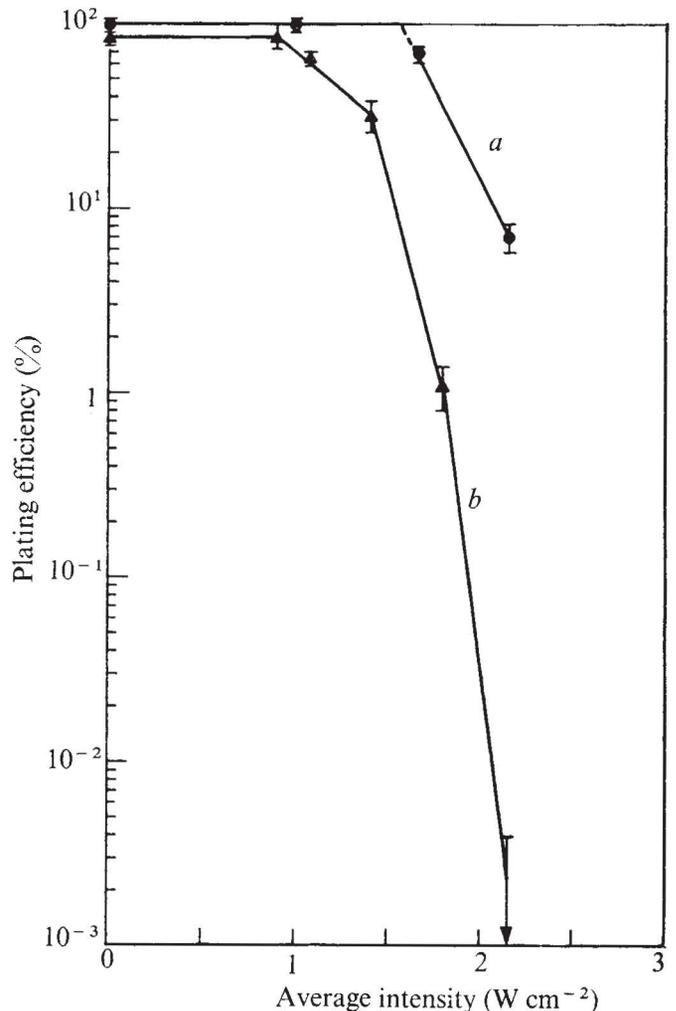
Fig. 1 Plating efficiencies (normalised to untreated controls) against US intensity. Cells were irradiated for 30 min at *a*, 37 °C; *b*, 43 °C. Error bars in this and the succeeding figures show s.e.m. Plating efficiency is defined as (no. colonies per dish/no. cells plated per dish) × 100. Cells plated per dish represents countable cells obtained by enzymic removal from Mylar after treatment.