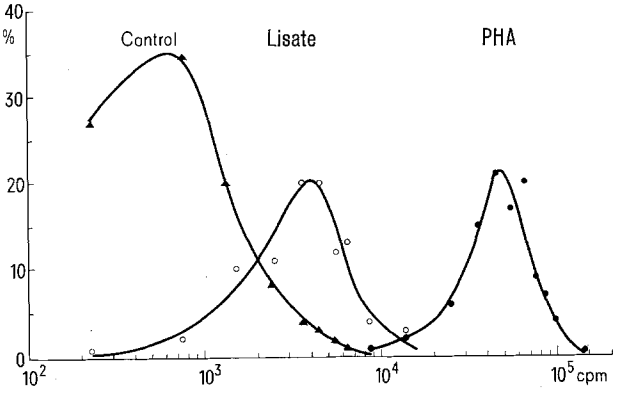
was done by density gradient sedimentation[4]. Bacterial hydrolysates[5] were prepared by digesting the endo- and exotoxins of a variety of established cultures of pathogenic bacteriae[6] with a protease of *B. subtilis* as described elsewhere[7]. The chemical composition was: oligopeptides 1.6 mg, polysaccharides 0.02 mg and nucleic acids 0.085 mg per 1 ml of hydrolysate. Lymphocytes were cultured in vitro for 110 h, followed by ^{3}H-thymidine labelling for additional 12–16 h, DNA-precipitation and liquid scintillation counting as described previously[8]. Separate lymphocyte cultures were stimulated with either 0.1 ml phytohemagglutinin (Difco) or 0.1 ml hydrolysate which in pilot studies at this concentration had its maximal stimulating effect. No stimulant was added to the control tubes. The cultures were done in triplicate, giving a total of 384 cultures for each mitogen. The results were expressed in cpm radioactivity of DNA precipitates. The data were statistically evaluated by an analysis of variance and a *t*-test adapted to multiple comparisons by DUNNET[9].

Results. The range of cpm was 367 to 2,138 for the controls, 1,583 to 9,357 for the hydrolysates and 20,445 to 120,830 for the PHA-cultures. The distribution of the counts is presented in the Figure. The difference between the background and each of the cultures stimulated by PHA and hydrolysate is significant at the level of $p < 0.01$.



Frequency distribution of counts per minute (cpm). Ordinate indicates % of all experiments done for stimulants or control.

Discussion. In contrast to PHA-P or bacterial endotoxins, the bacterial lysate is only a weak mitogen. Whether the mitogenic activity is due to the whole lysate as such, or to one or another of its components, remains to be shown. Because lymphocyte transformation occurred in almost 100% of the cultures it can be concluded that the substrate responsible for stimulation exerts an immunologically unspecific action. Further preliminary work suggests that this weak mitogen might be suitable for the demonstration of minor impairments of lymphocyte transformation in vitro.

Zusammenfassung. Hydrolysate, hergestellt mit einer *B.-subtilis*-Proteaseaus Endo-und Exotoxinen pathogener Mikroorganismen, stimulieren menschliche Lymphozyten in vitro. Die Stimulation ist schwach, verglichen mit Phytohämagglutinin, und wahrscheinlich immunologisch unspezifisch.

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Increased Immunogenicity of TSTA on Heterokaryocytes of Syngeneic Tumoral and Allogeneic Normal Cells

The immune response to tumour specific transplantation antigens (TSTA) is generally weak, thereby limiting both the efficiency of the spontaneous defence and the attempts of immunotherapy. Many efforts have been made in order to increase the immunogenicity of TSTA by means of aspecific adjuvants[1,2] or by the introduction of new antigenic determinants on tumour cells[3]. In 1967 SCHIERMAN and MCBRIDE[4] showed that a very weak humoral response to erythrocyte isoantigens was increased by the presence of stronger antigens on the same

Table I. Sequence of immunizing treatments and serological tests

Experiment 1						
Day 0 1st immunization 2×10^6 cells	Day 19 CI test	Day 25 Booster 2×10^6 cells	Day 33 Challenge 5×10^5 cells	Day 53 CI test	Day 67 CI test	Day 90 Final assessment of the number and volume of tumors
Experiment 2						
Day 0 Immunization 2×10^6 cells		Day 5 Challenge 5×10^5 cells		Day 20 CI test		Day 90 Final assessment of the number and volume of tumors

Table II. Number of homokaryocytes and heterokaryocytes in different fractions of the gradient after fusion of rat embryo fibroblasts with rat tumour cells[a]

	Homokaryocytes (%)[b]		Heterokaryocytes (%)	
	Labeled	Unlabeled		
After fusion	13.2	8.4	78.4[b]	12.6[c]
1st fraction	9.0	6.7	84.3	34.4
2nd, 3rd fractions	14.3	12.5	73.2	31.2
4th to 6th fraction	13.3	15.4	71.3	28.7
7th to 9th fraction	18.6	14.3	67.1	21.6
10th to 12th fraction	32.5	18.9	48.6	13.1
13th to 20th fraction	0	0	0	0

[a] Rat tumour cells were prelabeled with ^{3}H-thymidine and the number of heterokaryocytes was monitored by autoradiography[12]. [b] Percent of total number of polykaryocytes. [c] Percent of total number of cells. Fractions 1 to 9 (average value of heterokaryocytes 28.9%) were pooled and used for animal immunization.

cell. As to the cell-mediated immunity, DI MARCO et al.[5] demonstrated that the weak immunogenicity of a histocompatibility isoantigen was strengthened when it was placed on a hybrid cell which contained also histocompatibility alloantigens. In order to obtain this effect, the different antigens should be present in the same cell and the recipient host should be capable of reacting against the helper antigens. The aim of the present work was to increase the immunogenicity of syngeneic tumor cells by hybridization with allogeneic normal cells. Hybrids between tumour and normal cells have been used so far to study the relation of the cell genome to malignancy[6-8] or as a tool to inhibit the growth of cells from highly malignant tumours[9,10]. In this study we have followed two distinct experimental designs with different time intervals between sensitization and tumour challenge in order to detect cell-mediated immunity and the possible intervention of blocking antibodies (Table I).

Materials and methods. Tumour cells were obtained by trypsinization of a methylcholanthrene-induced sarcoma in inbred Fisher rats and normal rat fibroblasts by trypsinization of 17-day-old Wistar embryos. The techniques for the hybridization of rat tumour cells with normal rat embryo fibroblasts and for the separation of heterokaryocytes on a continuous calf serum gradient have been described[11,12]. Lower fractions of the gradient, containing predominantly polykaryocytes, were pooled and cells were treated with Mitomycin C (25 μg/10^6 cells) for 1 h at 37 °C, in order to avoid the growth of tumour cells without affecting their immunogenicity[2]. Two-month-old Fisher rats were immunized subcutaneously with 2×10^6 Mitomycin-inactivated cells and challenged with living tumour cells at different times after immunization. Colony inhibition tests were performed according to

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Table III. Tumour incidence

Group	Animals immunized with	No. of animals with tumours/ No. of animals inoculated	Animals with tumours (%)	Tumour volume[a]
Experiment 1				
1	T×N	9/12	75.0	3.0 ± 0.7
2	T	6/14	42.8	1.5 ± 0.3
3	N	6/10	60.0	2.7 ± 0.8
4	T + N	3/13	23.7	2.0 ± 1
5	T×T	0/10	0	0
6	—	14/15	93.0	4.9 ± 0.1
Experiment 2				
1	T×N	4/11	36.3	3.5 ± 0.9
2	T + N	11/12	91.6	3.7 ± 0.5
3	—	8/10	80.0	3.4 ± 0.6

T×N, hybrid cells obtained by fusion of rat tumour cells with normal rat embryo fibroblasts; T, rat tumour cells; N, normal rat embryo fibroblasts; T + N, mixture of rat tumour cells and normal rat embryo fibroblasts in equal amounts. [a] Tumour volume was calculated as the average size in cm of all tumours from each group of animals.

Table IV. Results of the tests of colony inhibition

Experiment 1						Experiment 2	
Test performed on day 19		Test performed on day 53		Test performed on day 67		Test performed on day 20	
	No. of colonies[a]		No. of colonies[a]		No. of colonies[a]		No. of colonies[a]
$T + S_1 + C'$	36 ± 1	$T + S_1 + C'$	29 ± 2	$T + L_5$	31 ± 7	$T + S_1 + C'$	107 ± 8
$T + S_5 + C'$	89 ± 3	$T + S_5 + C'$	109 ± 3	$T + S_1 + L_5$	96 ± 3	$T + S_2 + C'$	84 ± 3
$T + S_1$	90 ± 5	$T + S_6 + C'$	108 ± 4	$T + S_5 + L_5$	63 ± 1	$T + S_3 + C'$	79 ± 4
$T + S_5$	92 ± 1	$T + S_1$	123 ± 5	$T + S_6 + L_5$	75 ± 3	$T + S_1$	96 ± 8
$T + C'$	100 ± 2	$T + S_5$	111 ± 4	$T + L_6$	57 ± 1	$T + S_2$	101 ± 6
$T + L_1$	114 ± 6	$T + S_6$	99 ± 4	$T + S_1 + L_6$	186 ± 17	$T + S_3$	97 ± 1
$T + L_5$	60 ± 4	$T + C'$	83 ± 4	$T + S_5 + L_6$	78 ± 3	$T + C'$	115 ± 10
T	99 ± 8	$T + L_1$	62 ± 8	$T + S_6 + L_6$	109 ± 4	$T + L_1$	26 ± 2
		$T + L_5$	47 ± 3	$T + S_1$	108 ± 6	$T + L_2$	65 ± 10
		$T + L_6$	31 ± 1	$T + S_5$	120 ± 0	$T + L_3$	117 ± 9
		T	99 ± 2	$T + S_6$	102 ± 3	T	101 ± 6
				T	110 ± 7		

T, rat tumor cells; S_1 to S_6 and L_1 to L_6 correspond to sera and lymphocytes from groups of animals listed in table III; C', complement. Lymphocytes and sera were pools from blood of 4 animals from each group. [a]Average from 3 petri dishes.

HELLSTRÖM and HELLSTRÖM[13], using blood lymphocytes[14] when lymphocyte cytotoxicity was tested.

Results. The percentage of homo- and hetero-karyocytes in the immunizing inoculum, as determined by autoradiography[12], is shown in Table II. The incidence and volume of tumours are given in Table III. In the first experiment the highest values, close to those of controls, were detected in animals immunized with hybrid cells (group 1). The animals of all the other groups showed a lower take-in of tumours and the animals immunized with tumour cells fused to themselves (group 5) were completely resistant to tumour challenge. A test of colony inhibition performed on day 19 showed that sera from animals of group 1 and, at a lower degree, lymphocytes from animals of group 5 were cytotoxic (Table IV). 20 days after challenge (day 53) these effects were more remarkable and cytotoxicity was exhibited also by lymphocytes from animals of groups 1 and 6. The results of a test performed on day 67 indicate that serum of animals from group 1 inhibits the cytotoxic effect of lymphocytes.

In the second experiment, animals immunized with hybrid cells (group 1) showed a reduction in the incidence of tumours as compared to animals immunized with a mixture of non-fused normal and tumour cells (group 2) (Table III). In vitro tests performed on day 20 detected a remarkable cytotoxicity in lymphocytes from animals of group 1, while all sera were almost ineffective (Table IV).

Discussion. These results suggest that hybridization of tumour cells with allogeneic normal cells induces a relevant modification of TSTA immunogenicity. Indeed, the host response to a tumour isograft varies depending upon the manner of inducing sensitivity in the host, either with tumoral or hybrid cells. However, the schedule of immunization appears to be crucial for the anti-tumour response. When animals were immunized only once and challenged after a brief time interval, hybrid cells induced a resistance greater than that induced by a mixed population of non-fused tumoral and normal cells. Correspondingly, in vitro tests showed a solid cell-mediated immunity in animals immunized with hybrid cells, thus indicating that the presence of allogeneic and tumour antigens on the surface of the same cell increases the capability of TSTA to raise a cell-mediated reaction. On the contrary, when animals were immunized twice and challenged after a long time interval, hybrid cells induced the lowest degree of resistance and tumour incidence was close to that of non-immunized controls.

It seems unlikely that this effect could be due to a decreased immunogenicity of hybrid cells considering that a) the immunogenicity of hybrid cells is demonstrated by the resistance to tumour challenge in group 1 of experiment 2; b) if the immunogenicity of hybrid cells were low, the inoculum (containing 71% of T, N, T×T and N×N cells) should have produced a resistance close to the average resistance observed in the other groups of experiment 1; c) colony inhibition tests showed the presence of blocking antibodies. The appearance of such antibodies, probably as a consequence of the double immunization and of the long time interval between immunization and challenge, suggests that the high tumour incidence in group 1 of experiment 1 was due to a blocking effect. The fact that lymphocytes of these animals do not exhibit a high cytotoxicity for tumour cells (colony inhibition test on day 53) might depend consequently on a mechanism of central enhancement.

At present there is no explanation of the finding that tumour cells fused to themselves induced a complete anti-tumour resistance. One possible suggestion is that Mitomycin C as an alkylating agent binds to cellular proteins as well as to nucleic acids[15]. Therefore, it is possible that the drug interferes with the rearrangement of the membrane structures after fusion, contributing to the appearance of a new pattern of surface antigens. It should be noted also the resistance induced by normal embryo cells. This could be explained by sharing of antigenic determinants between tumour and embryonic cells[16].

In conclusion, these observations confirm the importance of the balance between cell-mediated and humoral immunity in the host response to tumours, as well as its

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variation in time and its dependence upon different schemes of immunization. The cell mediated immunity appears first; therefore there does exist a brief period when the reaction can be considered unidirectional; later, the humoral response may predominate. This finding should be taken into account in attempts to perform tumour immunotherapy in man[17].

[17] This investigation was supported by grants from the Consiglio Nazionale delle Ricerche, Rome. We thank Mr. L. FRANCHI for excellent technical assistance and Miss LUISA BENNI for useful advice.

Résumé. Une modification de l'immunogénicité des TSTA a été obtenue par l'hybridation des cellules d'un sarcome de rat (souche Fisher) avec des cellules normales allogéniques. La réponse des animaux syngéniques (immunisés avec les hétérokaryons) à l'implant de la tumeur fut strictement dépendante du schème d'immunisation.

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Alteration of Antigenic Components of Rat Parotid Gland Following Denervation

Removal of the innervation to salivary glands is followed by changes in the denervated and the normally innervated contralateral mate[1]. The characteristics of these changes depend however on the kind of denervation effected. Thus, the structural and functional modifications following removal of the superior cervical ganglion are not marked, while those following parasympathectomy are[2]. However, there are growth responses in the sympathectomized gland that are not found in the parasympathectomized gland[3]. These growth responses are attributed in part to circulating entities described nonspecifically as humoral factors[4,5]. It has recently been shown that removal of the superior cervical ganglion results in alteration of the immunoelectrophoretic pattern of the serum characterized by the appearance of additional antigenic bands[6]. The present work has been undertaken to determine whether these alterations were a reflection of antigenic changes in the gland itself.

Methods. Female Long-Evans rats, 6 months old, maintained on lab chow and water ad libitum were used in these experiments. Under light anesthesia, one of the following surgical procedures was performed: unilateral removal of the superior cervical ganglion (Sx); removal of a portion of the auriculotemporal nerve (Px); or a combination of these two procedures (PxSx); controls were either unoperated rats or those on which sham operations were done. In addition a group of unoperated rats was placed on a dietary regimen of liquid diet, Metrecal, (ME). 2 weeks after surgery or maintenance on liquid diet the paired parotid glands from animals of all groups were removed under Nembutal anesthesis (50 mg/kg, i.p.). The glands of each group were homogenized in saline, and the homogenate was then centrifuged at 10,000 rpm for 10 min and the supernatant was collected. Total protein content of the extracts was determined by the method of LOWRY et al.[7] and protein content of different extracts was adjusted to a concentration of 20 mg/ml.

Antisera were prepared by injecting saline extract of normal rat parotid gland s.c. into rabbits. The extract was incorporated into Freund's complete adjuvant for the first injection and into incomplete adjuvant for subsequent injections, as previously described[8]. Antiserum was absorbed with lyophilized rat serum in a concentration of 70 mg/ml of rabbit serum, which was found to be a proper concentration for neutralization by preliminary gel diffusion tests. Two-dimensional immunodiffusion was performed according to the method of OUCHTERLONY[9]. Immunoelectrophoresis was carried out according to the method of GRABAR and BURTIN[10] as modified by SCHEIDEGGER[11], using 1% 0.05 *M* barbital buffer at pH 8.6.

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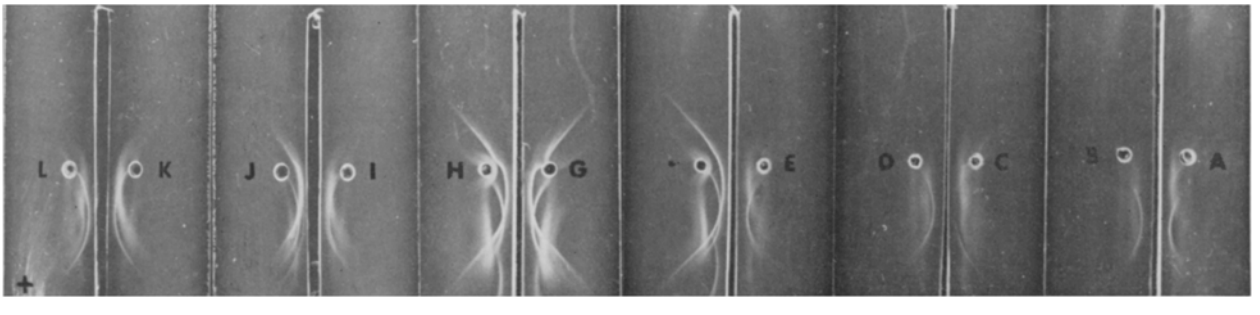

Immunoelectrophoresis in agar gel. Troughs contain rabbit anti-parotid gland extract absorbed with rat serum. Rat parotid gland extracts in wells (protein concentration 20 mg/ml): A) normal; B) parasympathectomized (Px); C) contralateral to parasympathectomized; D) parasympathectomized (Px); E) normal; F) sympathectomized (Sx); G) contralateral to sympathectomized; H) sympathectomized (Sx); (I) totally denervated (PxSx); J) contralateral to totally denervated; K) totally denervated (SxPx); L) maintained on liquid diet (ME).