Table 2. MICROSCOPIC DISTRIBUTION OF SPLEEN COLONIES IN MICE INJECTED WITH BONE MARROW FROM NORMAL (N) AND THYMECTOMIZED (Tx) DONORS

Donor source	No. of spleens examined	No. of colonies counted	Types of colonies				
			Erythroid	Granuloid	Mixed	Megakaryocytic	Undifferentiated
N	22	121	75 (62%)	16 (13%)	21 (17%)	2 (2%)	7 (6%)
Tx	58	108	84 (78%)	12 (11%)	7 (6%)	2 (2%)	3 (3%)

3×10^4. In spite of the differences in the yield of colonies in the different groups, bone marrow cells from thymectomized donors were found in every case to be less competent in inducing colony formation. The results of all the experiments (comprising 120 recipients of normal bone marrow cells and 115 recipients of bone marrow cells from thymectomized donors) are given in Fig. 1. The average number of colonies in recipient animals injected with 2×10^4 cells is 5·3 from normal donors and 2·5 from thymectomized donors. With the higher cell concentration (3×10^4) the averages were 7·8 and 3·2 respectively. These differences are significant at a 95 per cent confidence interval.

The results of microscopic examination of semi-serial sections of the spleens of animals from both the experimental and control groups are summarized in Table 2. Erythroid colonies were more frequent in the spleens of animals injected with marrow cells from thymectomized donors (84/108) than in those treated with marrow from intact mice (75/121). A reduction was also found in the number of mixed colonies in the spleens of the animals treated with bone marrow from thymectomized donors. In both cases, however, the differences were not significant.

Table 3. CELLULAR DISTRIBUTION IN THE BONE MARROW OF NORMAL AND THYMECTOMIZED C3H/eb MICE

Cell type	Normal	Thymectomized
Blasts	2·7 (1·7– 3·7)	7·1 (5·6– 8·6)
Promyelocytes and myelocytes	15·6 (11·6–19·6)	12·8 (7·8–17·8)
Metamyelocytes	11·0 (6·7–15·3)	11·4 (9·7–13·1)
Granulocytes	43·0 (31·8–54·2)	32·4 (23·6–41·1)
Young normoblasts	4·5 (2·0– 7·0)	5·6 (4·5– 6·7)
Adult normoblasts	17·3 (11·5–23·1)	22·3 (15·3–29·3)
Lymphocytes	4·9 (4·1– 5·5)	7·9 (4·7–11·1)
Plasma cells	0·3 (0·0– 0·8)	0
Mitotic cells	1·0 (0·98–1·04)	0·6 (0·01– 1·7)

The average values are derived from counts of 1,000 cells in each of five normal and five thymectomized animals. The figures in brackets represent 95 per cent confidence intervals.

Counts made from the bone marrow smears are summarized in Table 3. Young cells with a fine network of chromatin in the nucleus, a narrow rim of basophilic cytoplasm and which could not be classified in the myeloblastic or erythroblastic lines were considered to be blast cells. The most prominent change observed in the cellular distribution of the bone marrow of thymectomized mice was a significant augmentation in the number of blasts, from 2·7 in normal, to 7·1 in thymectomized, mice. Most of the other cell types were within the normal range, though there was a slight increase in the number of normoblasts and lymphocytes, which was compensated for by a reduction in the number of granulocytes. A few plasma cells were found in bone marrow, but were absent in the samples of thymectomized mice examined. The number of mitotic cells was smaller than that observed in normal mice, indicating that the bone marrow of thymectomized mice was less active than that of normal animals.

These experiments demonstrate that the cloning capacity of bone marrow cells of thymectomized mice is impaired in relation to that of intact controls. Because each spleen colony seems to be derived from a single multipotent bone marrow stem cell[8,9], these results suggest the existence of a regulatory function exerted by the thymus on the stem cell population of the bone marrow. A striking increase in the number of undifferentiated blasts was observed in the bone marrow of thymectomized mice. The same observation has been made in neonatally thymectomized Wistar rats[4]. We therefore conclude that the absence of the thymus leads to maturation arrest of bone marrow tissue, expressed morphologically by an increase in the number of blasts, and functionally by an impairment of bone marrow cloning capacity. Whether the thymus expresses its regulatory influence on the bone marrow cell population by a cellular or humoral mechanism, or both, is not yet known.

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Lymphocyte *in vitro* Cytotoxicity: Specific Release of Lymphotoxin-like Materials from Tuberculin-sensitive Lymphoid Cells

ALLOGRAFT immunity and delayed hypersensitivity reactions are caused chiefly by the action of host immune lymphoid cells[1]. Although these reactions have been much studied *in vivo* and *in vitro*, the mechanism of cell destruction is essentially unknown. There have been reports from this laboratory that a cell-free toxic factor released by immune and phytohaemagglutinin (PHA)-stimulated non-immune mouse lymphocytes is essential in the *in vitro* destruction of target L cells[2]. This factor, termed lymphotoxin (LT), caused *in vitro* cytolysis of both continuous and primary cells obtained from many animal species[3]. Later work showed that lymphocytes from various animal species, including man, could be stimulated *in vitro* to release LT, in the absence of target cells, by treatments which induce lymphocyte transformation, that is the mixed lymphocyte reaction, PHA and xenogeneic antibody[4]. It was therefore of interest to investigate whether lymphoid cells obtained from animals with delayed hypersensitivity to soluble antigens could be induced *in vitro* to release LT-like materials. We wish to report here that in the case of tuberculin hypersensitivity in mice, guinea-pigs and man, non-specific toxic materials are released when the cells are cultured in the presence of specific antigen.

Guinea-pigs and C57Bl/6 mice were sensitized by two subcutaneous injections, one in each flank, and an intraperitoneal injection, each containing 50 μg of tuberculin purified protein derivative (PPD) mixed in Freund's complete adjuvant. The injections were repeated after 2–3 months. Animals were killed 7 days after the final

injection and suspensions of splenic small lymphocytes were prepared by differential centrifugation as described earlier[2]. In addition, C57Bl/6 mice were immunized against DBA/2 antigens by repeated intraperitoneal doses of 0·2 ml. containing 40×10^6 DBA/2 spleen cells prepared as we have just described. Injections were given 3 days apart; the animals were killed 5 days after the fourth injection, and suspensions of splenic lymphocytes were prepared as described for the PPD treated animals.

Suspensions of human peripheral blood lymphoid cells were obtained from patients who gave specific positive skin reactions to PPD between 15 and 30 mm in diameter, and from tuberculin negative volunteers. Red blood cells were removed from the heparinized blood samples by sedimentation for 20 min at 37° C in an equal volume of 6 per cent citrated bovine fibrinogen[3]. The supernatant was carefully removed and the cells washed three times by alternate sedimentation (300*g* for 3 min) and resuspension in minimal essential medium. After the final washing, total and differential cell counts were made on living and May–Grünwald Giemsa-stained smears. The cells in these suspensions usually consisted of 20–35 per cent small lymphocytes, 40–45 per cent polymorphonuclear cells and 5–15 per cent monocytes.

The culture medium used in all experiments was Eagle's minimal essential medium (MEM) in Hank's salts supplemented with 0·2 μg/ml. of glutamine, 100 U/ml. of penicillin, 100 μg/ml. of streptomycin, 50 μg/ml. of mycostatin and 10 per cent foetal calf serum. Cells were cultured in screw capped tubes (15 × 150 mm) in 2·0 ml. of MEM containing 10×10^6 lymphocytes in an atmosphere of 95 per cent air and 5 per cent CO_2. The two soluble antigens used, PPD or bovine serum albumin (BSA), were suspended in MEM and added in a volume of 0·05 or 0·1 ml. to the test cultures. Cultures were incubated at 37° C for 72 h, after which the cells and debris were removed by centrifugation (1200*g* for 10 min) and passed through a 0·45 micron (pore size) 'Millipore' filter. The cell free medium was then tested for toxicity by adding 2·0 ml. to duplicate tubes of indicator L (mouse) and HeLa (human) cell monolayers (200,000 cells to a tube). Cultures were incubated at 37° C for 48 h during which time they were periodically examined microscopically, and finally cell viability was measured by the capacity of the cells to incorporate ^{14}C-amino-acids into cell protein[2].

Indicator cell cultures exposed to the cell-free medium obtained from sensitive lymphocytes cultured in the presence of antigen underwent cell destruction by 48 h. The cellular changes—an apparent weakening of the plasma membrane, blebbing and finally cytolysis—associated with destruction were characteristic of LT-induced cytotoxicity seen previously in other systems (refs. 3–5 and unpublished results of T. W. W. and G. A. G.). By contrast, indicator cells exposed to control medium grew normally and had normal morphology. The effect of the cell-free medium obtained from the various lymphocyte cultures on L and HeLa cell protein synthesis (viability) is shown in Table 1. Although the results shown are those of a single experiment, they are highly representative (within ± 10 per cent) of the data gathered from eight to ten separate experiments. Different human patients and new experimental animals were used in each experiment. Clearly, the specific antigen PPD stimulated the release of toxic materials from sensitive mouse, guinea-pig and human lymphoid cells. Stimulation was specific, for the same antigens did not induce release from normal or non-specific immune lymphocytes, and furthermore BSA failed to induce release from PPD-sensitive cells. Once released, however, cytolysis occurred by a non-specific mechanism, for both human and mouse indicator cells were affected.

There seem to be specific and non-specific ways of inducing *in vitro* release of lymphotoxins from immune and non-immune lymphocytes. Our results show that soluble antigen induced specific release from sensitive cells obtained from three animal species, and previous results demonstrated specific *in vitro* release when immune mouse lymphocytes contacted target cell antigens[2]. By contrast, both immune and non-immune lymphocytes obtained from these same animals can be induced to release lymphotoxins by various non-specific treatments—PHA, xenogeneic antibody and the mixed lymphocyte reaction (ref. 3 and unpublished results of T. W. W. and G. A. G.). These treatments, while chemically unrelated, have common properties; they all interact to some degree with the cell membrane and have been shown to induce lymphocyte transformation[5]. It is evident that lymphocytes must possess membrane receptors which when tripped by specific or artificial non-specific means result in "activation" and release of lymphotoxin. The relationship of lymphocyte transformation, characteristically defined by DNA synthesis and cell division, and what is referred to as "activation" remains to be clarified. The release of LT from PHA-stimulated human lymphocytes, however, is dependent on protein but not DNA synthesis[6].

Others have shown that lymphoid cells from tuberculin sensitive animals when cultured *in vitro* in the presence of antigen: (*a*) release soluble factor(s) which can cause inhibition of macrophage migration from capillary tubes[8]; (*b*) stop division of allogeneic and syngeneic rat fibroblasts[9]; and (*c*) cause non-specific cytolysis of xenogeneic red blood cells[9]. It is apparent that the initiating step(s) in these reactions is highly specific, but the effect on cells surrounding the stimulated lymphocytes is non-specific. Although the mechanism(s) of how cell migration and viability were effected in these systems was not demonstrated, the specific induction and release of non-specific cytolytic LT from sensitized lymphocytes on stimulation with antigen offer a direct explanation of these phenomena.

The work reported here provides evidence to support the concept that the cell destruction seen in allograft

Table 1. EFFECT OF CELL-FREE CULTURE MEDIUM OBTAINED FROM SENSITIVE AND NON-SENSITIVE LYMPHOID CELLS CULTURED IN THE PRESENCE OF PPD AND BSA ON THE VIABILITY OF INDICATOR L AND HELA CELL MONOLAYERS

Animal	Status	Treatment	Viability of indicator cells expressed as c.p.m. L (mouse)	HeLa (human)
Guinea-pig*	Non-immune	30 μg PPD	8,011	11,470
		60 μg PPD	7,756	12,410
		30 μg BSA	7,641	12,120
		60 μg BSA	7,781	11,200
		None	7,607	11,897
	Immune (PPD)	30 μg PPD	2,008	4,107
		60 μg PPD	1,831	3,810
		30 μg BSA	7,589	11,571
		60 μg BSA	7,707	12,001
		None	7,856	11,998
Mouse*	Non-immune	30 μg PPD	8,443	12,089
		60 μg PPD	8,673	11,970
		30 μg BSA	8,324	11,481
		60 μg BSA	8,287	12,203
		None	8,807	11,998
	Immune (PPD)	30 μg PPD	2,030	3,574
		60 μg PPD	3,500	4,891
		30 μg BSA	8,332	12,107
		60 μg BSA	8,401	11,872
		None	8,389	12,198
	Immune (DBA/2)	30 μg PPD	8,373	
		60 μg PPD	8,129	
		30 μg BSA	7,950	
		60 μg BSA	8,211	
		None	8,159	
Human†	Non-immune	10 μg PPD	16,947	20,011
		20 μg PPD	16,501	20,141
		20 μg BSA	16,707	19,948
		None	16,396	20,050
	Immune (PPD)	10 μg PPD	2,909	4,176
		20 μg PPD	3,076	3,989
		20 μg BSA	17,501	20,171
		None	16,947	20,279

Lymphoid cells were cultured for 72 h with PPD and BSA, cells and debris were removed and the medium was placed on fresh cultures of indicator cells. After incubation for 48 h, cell viability was assayed by measuring the capacity of the indicator cells to incorporate ^{14}C-amino-acids into cell protein.

* Each tube was labelled with 2·0 ml. of 0·5 μCi/ml. of ^{14}C-amino-acid hydrolysate for 15 min.

† Each tube was labelled with 2·0 ml. of 0·6 μCi/ml. of ^{14}C-amino-acid hydrolysate for 20 min.

immunity and delayed hypersensitivity *in vitro* may occur by a similar mechanism.

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Illegitimacy and Down's Syndrome

GERMAN recently suggested that the high prevalence of Down's syndrome among the children of older mothers results from relatively low frequencies of sexual intercourse among such mothers and a consequent increase in the probability of delay in fertilization of discharged ova[1]. To test this hypothesis, he suggested that the prevalence of Down's syndrome be studied among the offspring of unmarried mothers, "who as a group engage in coitus sporadically rather than systematically". On similar grounds, Goodhart[2] postulated that a particularly high prevalence should be expected in the children of young unmarried mothers.

In response to these suggestions, Shokeir[3] has reported that, among thirty-one cases of Down's syndrome seen for genetic counselling, nine were illegitimate children born to mothers below the age of 20.

Other substantial grounds for rejecting German's general hypothesis have been presented[4,5], but no estimate of the frequency of Down's syndrome in illegitimate children seems previously to have been made, so it seemed worthwhile to provide this for a series of cases recently compiled for other purposes.

By searching all known sources, I have assembled a series of 1,810 cases of Down's syndrome among white children born to mothers residing in Massachusetts between 1954 and 1965. The estimate of prevalence obtained—1·41 per 1,000 live births—is one of the highest found in any American survey and suggests that ascertainment was reasonably complete.

Birth certificates were obtained for all but four of these children and these provided information on the age of the mother. Legitimacy is not directly reported on birth certificates in Massachusetts. An inferential method of ascertaining legitimacy—used by the US National Center for Health Statistics[6]—was therefore adopted. This method is known to overstate slightly the number of illegitimate births, but the error is small (0·7 per cent total overstatement among the white population of the United States)[6] and, in the context of the present results, in a conservative direction.

According to this method, children are classified as follows.

Status	Child's surname	Father's surname	Mother's maiden name
Legitimate	A	A	B (or A)
Illegitimate	A	—	A
	A	—	B
	A	B	A
	A	B	C

Table 1 gives the distributions of live births, mongol births and inferred illegitimate mongol births by age of mother. Twenty-seven (14·9 per 1,000) of the children with Down's syndrome were classified as illegitimate. The illegitimacy ratio among these children shows the same trends with increasing maternal age as among children in general.

Table 1. NUMBER OF LIVE BIRTHS, CHILDREN WITH DOWN'S SYNDROME, NUMBER INFERRED TO BE ILLEGITIMATE, AND ILLEGITIMACY RATIOS, BY MATERNAL AGE. MASSACHUSETTS WHITE POPULATION, 1954–65

Age of mother	Total No. of live births	Down's syndrome No. of live births	Down's syndrome No. illegitimate	Illegitimate per 1,000 live births Down's syndrome	Illegitimate per 1,000 live births 32 states†
<15	378	—	—	—	453·9
15–19	93,163	57	4	70·2	70·7
20–24	388,743	240	5	20·8	21·9
25–29	378,887	266	6	22·6	11·5
30–34	258,073	295	4	13·6	10·4
35–39	133,236	476	3	6·3	12·9
40–44	33,304	416	5	12·0	15·8
45+	1,662	52	—	—	
Unknown	—*	8	—	—	—
Total	1,287,446	1,810	27	14·9	16·1‡

* Births of unknown maternal age are distributed according to the distribution of the known births.
† Thirty-two states reporting to the National Center for Health Statistics[6]; white births, 1960.
‡ Total is standardized to the maternal age distribution of the children with Down's syndrome.

No entirely satisfactory data are available for comparing the illegitimacy ratio among the Down's syndrome cases with that in an unaffected group of children, because these data are neither recorded nor reported in Massachusetts. For thirty-two other states, however, data have been assembled by the US National Center for Health Statistics[6]. For the white population of these states, the data for the central year of birth of the Down's syndrome cases are given in Table 1. The two sets of ratios are fairly similar. The data on the individual states, given in the report quoted, indicate that, for the white population, states in the north and east parts of the country tend to have somewhat higher illegitimacy ratios than the national average. There is therefore no evidence here of a high frequency of illegitimacy among the children with Down's syndrome.

Estimates of prevalence rates of Down's syndrome by legitimacy status are given in Table 2. The number of illegitimate live births was estimated by applying, in each year of birth and maternal age group, the illegitimacy ratios reported for the white population of the thirty-two reporting states. Both legitimate and illegitimate births show increasing prevalence of mongolism with advancing maternal age. In the younger maternal age groups, the prevalence of mongolism is almost identical in the illegitimate and legitimate births. In the two age groups over 35, rates are somewhat higher in the legitimate births. These differences are not statistically significant. Overall,

Table 2. NUMBER OF LIVE BIRTHS, CHILDREN WITH DOWN'S SYNDROME, AND PREVALENCE RATES, BY MATERNAL AGE AND LEGITIMACY. MASSACHUSETTS WHITE POPULATION, 1954–65

Age of mother	Number of live births Legitimate*	Number of live births Illegitimate*	Number with Down's syndrome Legitimate*	Number with Down's syndrome Illegitimate*	Cases of Down's syndrome per 1,000 live births Legitimate	Cases of Down's syndrome per 1,000 live births Illegitimate
<15	226	152	—	—	—	—
15–19	86,169	6,994	53	4	0·62	0·57
20–24	379,465	9,278	235	5	0·62	0·54
25–29	374,396	4,491	260	6	0·69	1·34
30–34	255,303	2,770	291	4	1·14	1·44
35–39	131,540	1,696	473	3	3·59	1·77
40+	34,388	578	463	5	13·46	8·65
Unknown	—	—	8	—	—	—
Total	1,261,487	25,959	1,783	27	1·41	1·31†

* See text for methods of estimation.
† Total standardized to the maternal age distribution of the legitimate births.