antibody. Because it was not possible to differentiate intracellular HSF and HSF-anti HSF complexes morphologically in this system and because there was no significant difference in the intracellular distribution of HSF, whether presented as the antigen alone or as a soluble complex, the explanation for these quantitative findings remains unknown. Whether the increased uptake in the presence of increasing amounts of antibody is the result of complex size or bears a specific relation to the antibody component of the globulin is uncertain.

Ferritin is known to enter cells readily by pinocytosis and perhaps also by a more rapid mechanism similar to pinocytosis(13). Presumably soluble ferritin-antiferritin complexes enter even more rapidly, as large molecular weight complexes. The possibility remains, however, that the complex, having activated the cell membrane, dissociates into component parts which separately are incorporated into the cell.

The difference noted between degree of uptake of rabbit or chicken complexes by guinea pig cells suggests the possibility of a specific difference in the recognition of complexes prepared with antisera of different species of phagocytic cells.

Summary. The ingestion of soluble ferritin-antiferritin complexes was studied by parallel immunofluorescent, electron microscopic and isotope trace labeled antigen methods. The complexes were ingested by guinea pig spleen cells or mouse peritoneal macrophages. The degree of cellular activity demonstrated by these techniques was dependent on the degree of antigen excess used in preparation of the complexes, the source of antisera and the particular methodology applied.

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Influence of Oligodeoxyribonucleotides on Early Events in Antibody Formation.* (30276)

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Oligodeoxyribonucleotides, present in enzymatic digests of DNA, can enhance bacterial population changes(1,2,3), can cause a selective stimulation of DNA synthesis in "resting" Gram-positive bacteria(4), and can restore immune responses in irradiated animals(5) as well as in animals made nonresponsive to a specific antigen by administration of 6-MP(6). These effects are independent of the source of DNA employed in preparation of the oligodeoxyribonucleotides. Oligonucleotides can also produce an occasional elevation of circulating antibody levels in normal, mature animals exposed to antigen plus DNA digest(7,8), or antigen plus RNA digest(8), and they can increase host-resistance to a variety of Gram-negative pathogens provided the oligonucleotides are ad-

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ministered in conjunction with a very small amount of bacterial endotoxin (cf. 7). Studies with bacteria have indicated that the size of the active oligodeoxyribonucleotides is relatively small (2 to 5 nucleotides) and that larger fragments can interfere with the activity of the small fragments(3).

The data to be reported here indicate that the stimulatory effects of oligodeoxyribonucleotides on antibody formation in normal, mature animals involve early events and require concurrent administration of specific antigen.

Methods. Sheep red cells served as antigen and were injected i.v., at a concentration of 1×10^8/mouse, into 20-25 g AKR females. DNA digests were prepared by exposing calf thymus DNA (Worthington 400 mg/400 ml biosaline) to pancreatic DNAase (Worthington, 100 mg/70 ml biosaline + 30 ml 0.1 M Mg^{++} SO_4) for 24 hours at 37°C; the digests were then sterilized by Millipore filtration, and usually supplemented with deoxyribonucleosides (400 mg/100 ml biosaline of each of the 4 common nucleosides) and yeast RNA (Schwartz, 400 mg/100 ml biosaline). The final concentrations per ml of the usual preparation, therefore, contained 400 γ DNA, 100 γ DNAase, 400 γ of each of the deoxynucleosides, < 400 γ yeast RNA (yeast RNA is poorly soluble). The preparations, when checked, were non-pyrogenic for rabbits. The digest, with or without supplement, was routinely administered, in quantities of 0.5 ml i.v. and 1.0 ml i.p., at time of antigen administration, and, in certain experiments, at intervals thereafter. Immune responses were measured by the technique of localized hemolysis in agar(9) using spleen cells from animals sacrificed at 1, 2, 3 etc. days following the first administration of antigen. Assays were routinely conducted on 1/5 of a cell suspension prepared from an entire spleen. The total number of spleen cells was determined by direct counting in a hemocytometer, but they did not increase dramatically above the norm (0.8-3 $\times 10^8$) in any of the tests here reported. The endotoxin (Difco) employed in some of the tests was derived from *Serratia marcescens* and was administered intravenously. Groups of 5 animals were used in all spleen cell assays.

Results. As shown in Tables I, II, IV, V and VII, the usual number of hemolysin-forming spleen-cells in non-immunized animals ("background") was approximately 10-30 per spleen. As illustrated in Tables I, III, IV, V, and VII, this number becomes significantly higher 48 hours following administration of specific antigen (sheep red cells) or endotoxin(3,10,11). The number of hemolysin-forming spleen cells is not elevated when oligodeoxyribonucleotides (= oligonucleotides, DNA + DNAase, or DNA digest) are administered *without* specific antigen, but when they are administered *with* specific antigen (Table I) an increase, significantly above that caused by antigen alone, is produced within 48 hours after immunization. Oligo-

TABLE I. Influence of DNA Digest on the Number of Hemolysin-Producing Spleen Cells 48 Hours After Immunization.

Treatment of spleen donors	Exp	No. of hemolysin-producing cells per 1/5 spleen after 48 hr #21*	#22*	#40†
None		1	7	12
		2	6	3
		2	3	5
		6	2	6
		2	1	2
	Avg	2.6	3.8	5.6
Sheep red blood cells 10^8/animal (given once only)		24	16	83
		35	17	72
		27	19	71
		36	28	44
		13	8	54
	"	27.0	17.6	65.0
Sheep red blood cells + DNA digest		49	57	89
		57	62	91
		81	58	169
		133	56	102
		46	57	117
	"	73.2‡	58‡	113.6‡
DNA digest		—	5	10
		—	4	5
		—	1	7
		—	2	4
		—	1	9
	"		2.6	7.0

* DNA digest preparation #51 administered at 0 and 24 hr, antigen at 0 hr only.

† DNA digest preparation #55 administered at 0 and 24 hr, antigen at 0 hr only.

‡ The difference between these values and those obtained in the absence of DNA digest are statistically highly significant ($2.5\% > p > 1.0\%$).

TABLE II. Influence of Endotoxin (*S. marcescens*) on Number of Hemolysin-Producing Spleen Cells 48 Hours After i.v. Administration of Endotoxin.

Treatment of spleen donors	Avg No. (± S.E.) of hemolysin-producing cells per 1/5 spleen after 48 hr
None	6.3 ± 1.1
Endotoxin 5γ/mouse	152.8 ± 12.3
" 10γ/ "	212.2 ± 26.4
" 5γ + DNA digest	242.6 ± 17.8
" 10γ + " "	450 ± 61.5

Endotoxin given at 0 hr only, DNA digest at 0 and 24 hr.

nucleotides also enhance the effects produced by endotoxin in the absence of specific antigen (Table II). It was ascertained that oligonucleotides stimulate immune responses only when given to the spleen donors; addition of DNA digest to the suspension of spleen cells and red blood cells at time of assay in agar has no influence on plaque numbers.

When, in experiments with sheep red cells, oligonucleotides are given more than once, namely when a total of 6 ml of DNA digest is injected at 0, 6, 24 and 30 hours following antigen administration, the stimulation is no greater than that produced by one injection of 1.5 ml of DNA digest at 0 hour (Table III). However, when multiple injections of DNA digest are accompanied by a small amount of specific antigen (*i.e.*, when 1% of the original antigen dose is given with DNA digest at 6, 24 and 30 hours), an enhancement far in excess of that obtainable by one injection of DNA digest is produced. These data indicate that oligonucleotides stimulate only when administered in conjunction with specific antigen (the latter being replaceable by endotoxin). Note that in the absence of DNA digest, multiple injections of antigen alone (10^8 sheep red blood cells followed by 10^6 sheep red blood cells/mouse) do not cause a spleen cell response significantly different from that obtainable following a single injection of antigen.

TABLE III. Influence of Repeated Administration of DNA Digest, With or Without Specific Antigen, on Number of Hemolysin-Producing Spleen Cells.

Treatment of spleen donors	Avg No. (± S.E.) of hemolysin-producing cells per 1/5 spleen after 48 hr
10^8 sheep red blood cells/mouse	19.2 ± 3.3
10^8 sheep red blood cells + 10^6 sheep red cells (3×) at 6, 24, and 30 hr	24.2 ± 2.7
10^8 sheep red blood cells + DNA digest (1×)	53.6 ± 10.2
10^8 sheep red blood cells + DNA digest (4×)	57.4 ± 8.9
10^8 sheep red blood cells + 10^6 sheep red cells (3×) + DNA digest (4×)	129.6 ± 12.5

The stimulation of antibody formation following administration of specific antigen plus DNA digest affects principally early events. Table IV lists average numbers of hemolysin-forming spleen cells, collected by assays on 5 spleens per group, and it can be seen that in the case of sheep red blood cells as antigen the optimal difference between animals exposed to antigen plus oligonucleotides *vs* animals exposed to antigen alone occurs 48 hours after the initial antigen exposure. These differences become less pronounced, or even nil, 72 and 96 hours after initial antigen administration.

Supplementation of the DNA digest preparation with deoxynucleosides and RNA appears unnecessary since the effects are identical when DNA digest is given without these supplements. A dosage of 600 γ DNA + 150 γ DNAase appears to be close to optimal as indicated by the data in Table V. The actual extent of stimulation by DNA digest occasionally can differ with different lots of mice and complement despite the use of the same preparation of DNA digest. However, relative differences between DNA digest-treated groups and untreated control groups tend to remain similar.

A significant stimulatory oligonucleotide effect is demonstrable with spleens removed 48 hours after administration of antigen plus DNA digest even when the amount of antigen is reduced from 10^8 sheep red cells per mouse i.v. to 10^7 per mouse. When the antigen dosage is reduced to 10^6 red cells per mouse, no immune response above background (= 12 cells/spleen) is obtained 48 hours later in untreated animals, but an elevation (= 53 cells/spleen) occurs in DNA digest-treated animals.

TABLE IV. Extent of the DNA Digest Effect at Various Times Following Immunization of AKR Mice with Sheep or Horse Red Blood Cells, and Influence of Routes of Administration. (Italicized figures represent values that in t test proved to be significantly different from corresponding values obtained in absence of DNA digest.)

Treatment of spleen donors	Avg No. of hemolysin-producing cells per 1/5 spleen after 24 hr	48 hr	72 hr	96 hr
Sheep red blood cells (10^8/mouse)	—	62.4	307.8	—
" " " " + DNA digest #45*	—	*184.8*	*491.2*	—
" " " "	4.2	16.0	234.8	4580
" " " " + DNA digest #44†	4.0	*44.8*	328.0	4560
Sheep red blood cells	—	35.2	1408	5006
" " " " + DNA digest #50† i.v./i.p.	—	*81.4*	1890	—
" " " " + " " i.v.	—	44.8	2130	5880
" " " " + " " i.p.	—	*60.8*	1960	8202
" " " " + 2/3 DNA digest i.v.	—	*60.8*	1676	—
" " " " + 1/3 " " "	—	50.8	1740	—
Horse red blood cells (10^9/mouse)	—	3.6	13.8	80.4
" " " " + DNA digest #51†	—	7.8	19.8	*161.0*
None	—	—	—	1.4

* Given 4 times with 10^6 sheep red blood cells.
† Given only once in conjunction with red blood cells.

The route of administration of oligonucleotides influences the extent and time of stimulation as illustrated in Table IV.

A 24-hour delay in the administration of oligonucleotides, *i.e.*, injection of DNA digest 24 hours after antigen, still results in a significant stimulation of early responses as revealed by spleen assays 48 hours after antigen administration (for example, average, per 1/5 spleen, for mice receiving antigen only: 44; DNA digest + antigen given at 0 hr: 96.0; DNA digest given 24 hrs after antigen: 94.6).

Oligonucleotides also stimulate early stages of the secondary response, but as might have been anticipated, all events occur more rapidly than during the primary response. Table VI shows an optimal influence of DNA digest on the secondary response 24 hours after the second administration of antigen and a disappearance of a detectable difference 48 hours after the second antigen injection.

TABLE V. Influence of Different Dosages of DNA Digest on Number of Hemolysin-Producing Spleen Cells.

Treatment of spleen donors	Avg No. of hemolysin-producing cells per 1/5 spleen after 48 hr
None	2.6 ± .9
Sheep red blood cells	27.0 ± 4.2
Sheep red blood cells + 1200γ DNA digest (prep #51)	92.0 ± 6.3
Sheep red blood cells + 600γ* DNA digest (prep #51)	73.2 ± 16.1
Sheep red blood cells + 300γ DNA digest (prep #51)	45.4 ± 4.5
Sheep red blood cells + 150γ DNA digest (prep #51)	31.2 ± 6.7

* Usual dosage, administered at 0 and 24 hr.

In the case of previously investigated bacterial systems the active components in the DNA digest were found in the dialyzable oligonucleotide fractions; the non-dialyzable portion of the DNA digest failed to produce any significant stimulatory effects (cf. 3). However, in the tests described here with mice, the non-dialyzable portion of the digest also shows activity, provided the concentration of 260-absorbing materials is adjusted to the usual concentration of such materials in non-dialyzed preparations. This suggests that either (a) large DNA fragments can stimulate mammalian cells, or (b) following injection of a sufficiently high concentration of large, non-dialyzable DNA fragments, these may be broken down into active oligonucleotides by the animal's nucleases. In view of the results with bacteria, the last cited interpretation would appear to be the more likely explanation of the results obtained with mice. As in the case of bacterial test systems, a mixture of mononucleotides, adjusted to the concentration of nucleotides

TABLE VI. Influence of DNA Digest on Secondary Response.

Treatment of spleen donor at time of 2nd antigen exposure	Avg No. (± S.E.) of hemolysin-producing cells per 1/5 spleen after 6 hr	12 hr	24 hr	48 hr	72 hr
None*	—	—	900 ± 52.3	—	—
Sheep red blood cells (10^8/mouse)	—	—	900 ± 114.8	3136 ± 148.8	5460 ± 966.5
" " " " + DNA digest #51	—	—	1500 ± 278.4	2816 ± 350.0	4170 ± 854.9
None†	238 ± 30.3	—	—	170 ± 29.3	—
Sheep red blood cells (10^8/mouse)	366 ± 53.0	274 ± 42.5	424 ± 42.8	2704 ± 414.3	—
" " " " + DNA digest #54	332 ± 58.4	328 ± 44.8	928 ± 82.8	2548 ± 475.9	—
None†	—	—	142 ± 38.8	—	—
Sheep red blood cells (10^9/mouse)	—	—	552 ± 77.3	—	—
" " " " " " + DNA digest #53	—	—	704 ± 133.3	—	—
Sheep red blood cells (10^8/mouse)	—	—	336 ± 52.1	—	—
" " " " " " + DNA digest #53	—	—	920 ± 208.7	—	—
Sheep red blood cells (10^7/mouse)	—	—	188 ± 34.3	1330 ± 116.7	—
" " " " " " + DNA digest #53	—	—	302 ± 15.4	1262 ± 152.7	—

* All mice in this group received 10^8 srbc/mouse 10 days earlier. DNA digest given at 0 hr only.
† *Idem* 12 days earlier.

in active DNA digest preparations, or a mixture of mononucleosides, or oligonucleotides derived from yeast RNA and mouse spleen RNA (kindly supplied by Dr. E. Cohen) fail to produce stimulatory effects comparable to those elicited by the oligodeoxyribonucleotides. The active materials in the DNA digest solution are resistant to boiling and are unaltered in their activity following repeated (6 times) freezing and thawing; however, presumably as a result of continued enzymatic activity, they lose their activity upon standing at room temperature for several days.

Kinetin riboside, as well as high levels of kinetin, are known inhibitors of DNA digest effects in the bacterial tests systems(12,13); both the oligonucleotide-elicited stimulation of DNA synthesis in "resting" bacterial cells as well as the enhanced population changes in cultures of *D. pneumoniae* and *B. abortus* are prevented in their presence. It has now been ascertained that these compounds, at a dosage of 2 mg/mouse, also inhibit the usual DNA digest effect on early antibody formation without interfering with the basic non-stimulated immune response (Table VII). As in the bacterial work, low levels of these compounds (0.2 mg/mouse) produce a modest, but statistically significant, stimulation in the absence of DNA digest.

Injection of Actinomycin D at time of antigen administration depresses the immune response in the absence and presence of oligonucleotides (Table VII).

A stimulation of early immune responses by oligodeoxyribonucleotides also occurs when chicken- or horse- red blood cells serve as antigens. However, associated with the lesser immunogenicity of the latter for mice, is a delay in the optimal difference between spleen cell assays of DNA digest-treated and non-treated animals; this difference is optimal at 48 hours following immunization with sheep red blood cells, but optimal at 96 hours following immunization with horse red blood cells (Table IV). No significant cross-reactivity between horse- and sheep- red cells has been detectable. A test system employing lysis of bacteria instead of red cells as indicator of antibody formation by spleen cells (14), has revealed that the oligodeoxyribonucleotides also can cause a significant increase in the early number of spleen cells forming antibodies against *Escherichia coli* (14).

Discussion. The foregoing assays on spleen cell populations show that enzymatic digests

TABLE VII. Influence of Kinetin Riboside and Actinomycin D on Hemolysin Production by Spleen from DNA Digest-Treated and Untreated Mice.

Treatment of spleen donors*	Avg No. (± S.E.) of hemolysin-producing cells per 1/5 spleen after 48 hr
Sheep red cells	17.6 ± 3.2
Idem + DNA digest #51	58.0 ± 1.0
" + kinetin riboside (2 mg/mouse)	14.6 ± 2.6
" + " " + DNA digest	15.0 ± 2.5
" + kinetin (2 mg/mouse)	19.2 ± 1.5
" + " + DNA digest	24.4 ± 5.6
None	6.4 ± 2.1
Sheep red cells	43.8 ± 5.1
Idem + .5 γ Actinomycin D	27.0 ± 4.4
" + 2.5 γ " "	10.4 ± 2.6
" + 10 γ " "	1.8 ± .9
" + DNA digest #54	93.8 ±13.3
" + " " + .5 γ Actinomycin D	48.4 ± 9.9
" + " " + 2.5 γ " "	17.4 ± 2.7
" + " " + 10 γ " "	.8 ± .5

* All materials, including red cells (10^8 at 0 hr, 10^6 at 24 hr), were administered at 0 and 24 hr.

of DNA, and presumably the oligodeoxyribonucleotides therein, can significantly increase the early number of hemolysin-forming, and/or hemolysin-releasing, spleen cells (*hfc*). Preliminary tests, in which each spleen was cut into 24 pieces, and each piece assayed separately for numbers of *hfc,* indicate that the stimulation involves a more rapid multiplication of early arising, or early activated, hemolysin-forming clones. This is indicated by the fact that the early number of *hfc* is not equally elevated in all spleen pieces, but rather that the spleen from stimulated animals contains some pieces with unusually high numbers of *hfc* and other pieces with the usual number of *hfc.* (Studies to be reported separately have shown (1) that the distribution of *hfc* is non-random throughout the spleen, and (2) that this non-randomness is apparently not due to an anatomical feature.) The fact that the stimulation by oligodeoxynucleotides involves principally early immune responses suggests that stimulators akin to, or identical with, the oligodeoxynucleotides, may be released naturally some time after the initiation of a specific immune response, possibly as the consequence of antigen-antibody reactions (cf. 7). This may account for the difficulty of detecting oligonucleotide effects in assays on circulating antibodies(11); by the time that the latter can be measured, the early stimulation, due to injected nucleotides, may be masked by the subsequent natural release of stimulators.

It is interesting to note that the stimulatory effects of oligonucleotides require concurrent administration of specific antigen, whereas comparable stimulation by endotoxin can be elicited without injection of specific antigen. It could be argued that endotoxin may release specific antigen, from storage sites, or cross-react with it. However, the latter is unlikely in view of the fact that immunization of mice with sheep red cells does not increase plaque counts in tests on localized immune lysis of *Escherichia coli* 0127 in agar(14), and in view of the diversity of specific antigens that can be replaced by endotoxin. Thus, endotoxin (from *Serratia, E. coli,* etc.) is active in the absence of added specific antigen in increasing host resistance to Gram-negative pathogens(15) whereas DNA digest can produce similar protective effects only when administered with a very small amount of specific antigen, *i.e.,* with 100 heat-inactivated cells of the bacterial pathogen that is employed subsequently for challenge (Fukui and Braun, unpublished). The need for specific antigen in the stimulation of immune responses by oligonucleotides, therefore, may be due to a requirement for another factor that is automatically provided by endotoxin. Altered cell permeability is one candidate for such a factor. Endotoxin,

which is believed to cause a release of stimulatory oligonucleotides from intracellular sites(7) may, by virtue of its multitude of physiological effects(16), also initiate a simultaneous alteration in permeability prerequisite for the entrance of the oligonucleotides into antibody-forming target cells. In the case of the antigen-requiring oligonucleotide effect this altered permeability of target cells may be achieved as a result of a specific antigen-antibody reaction. If these considerations are correct, it may prove possible to substitute a permeability-altering agent for specific antigen in the elicitation of the oligonucleotide effect. Studies in this direction have been initiated and early results indicate that, in the absence of specific antigen, DNA digest can produce stimulatory effects when given with chlorpromazine.

The present data strengthen the previously expressed belief(3,7) that the activity and multiplication of antibody-forming cells under natural conditions may be influenced by cell breakdown products, and that such effects may be exaggerated following administration of cytotoxic adjuvants.

Summary. The number of hemolysin-forming cells, assayed by Jerne's technique in spleens removed from AKR mice, 48 hours after immunization with heterologous red cells, is significantly higher when antigen is administered in conjunction with an enzymatic digest of calf thymus DNA. This stimulation by oligodeoxyribonucleotides is not matched by comparable effects of oligoribonucleotides; mixtures of monodeoxyribonucleotides or -sides are inactive. The stimulation appears to involve a stimulated multiplication of early appearing, or early activated, antibody-forming clones, and is more difficult to discern as the interval between immunization and assay of spleen cell populations increases. Oligodeoxyribonucleotides do not stimulate early immune responses unless specific antigen is administered concurrently; possible reasons for this requirement of antigen, in contrast to a lack of such requirement in comparable stimulations produced by bacterial endotoxins, are considered. The influence of dosage and route of administration of DNA digest have been analyzed and an effect on secondary as well as primary responses has been demonstrated. Kinetin riboside abolishes the stimulatory effects of oligodeoxyribonucleotides without influencing the basic, non-stimulated immune response, whereas Actinomycin D interferes with the immune response in the absence and presence of oligodeoxyribonucleotides. Possible relationships to problems of natural and adjuvant-elicited stimulations of antibody production have been discussed.

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