Review

Review: 2-Mercaptoethanol alteration of in vitro immune functions of species other than murine ☆

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ABSTRACT

Descriptions that organosulfurs could alter biologically relevant cellular functions began some 40 years ago when cell mediated and humoral murine in vitro immune responses were reported to be dramatically enhanced by any of four xenobiotic, sulfhydryl compounds— 2-mercaptoethanol (2-ME), dithiothreitol, glutathione, and L-cysteine; the most effective of the four was 2-ME. These findings triggered a plethora of reports defining 2-ME benefits for a multitude of immunological processes, primarily with murine models. This led to investigations on 2-ME alterations of (a) immune functions in other species, (b) activities of other cell-types, and (c) in situ diseases. In addition, the early findings may have been instrumental in the identification of the previously undefined anticarcinogenic chemicals in specific foods as organosulfurs. Outside the plant organosulfurs, there are no comprehensive reviews of these areas to help define mechanisms by which organosulfurs function as well as identify potential alternative uses. Therefore, the present review will focus on 2-ME alterations of in vitro immune functions in species other than murine; namely, fish, amphibian, reptile, avian, whales, dolphins, rat, hamster, rabbit, guinea pig, feline, canine, porcine, ovine, bovine, and human. Processes, some unique to a given species, were in general, enhanced and in some cases dependent upon the presence of 2-ME. The largest benefits occurred in media that were serum free, followed by those in autologous serum and then fetal bovine serum supplemented medium. Concentrations of 2-ME were generally in the low μM range, with exceptions of those for salamander (20 mM), turtles (70 mM) and dolphins (7 mM). The few studies designed to assess mechanisms found that changes induced by 2-ME were generally accompanied by alterations of reduced/oxidized glutathione cellular concentrations. The major benefit for most studies, however, was to increase the sensitivity of the culture environment, which permitted a specific process to be more easily dissected.

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Contents

1. Introduction

It has been some 40 years since we first reported (Heber-Katz and Click, 1972; Katz-Heber et al., 1973; Peck and Click, 1973a; Peck et al., 1973) that murine cell mediated immunological functions could be replicated in vitro under conditions that closely approximated those in situ, namely in culture medium not supplemented with heterologous sera. The dramatically enhanced responses were found to depend upon supplementation of a newly defined culture medium (Click et al., 1972b) with any of four xenobiotic organosulfhydryls—2-mercaptoethanol (2-ME), dithiothreitol (DTT), reduced glutathione (rGSH), and L-cysteine (Cys); the most effective of these four was 2-ME. Not surprisingly, these findings led to an extensive number of investigations (primarily with 2-ME) on benefits for, and mechanisms of, 2-ME on many different aspects of murine immune functions. Because of the enormous numbers of reports (>1000 in PubMed) alteration of murine functions will not be included here. Suffice it to say that essentially any in vitro function was found to benefit and in some cases was found to absolutely depend upon its presence. Regrettably, in many of these publications, the literature cited (Chen and Hirsch, 1972a, 1972b) as the origin of 2-ME's dramatic enhancement is totally incorrect. The original research presented in 1971 at the First Congress of Immunology (workshop #71) in Washington, DC (Click, 1971) was at the time in press and occurred in print in early 1972 (Click et al., 1972a, 1972b).

There seems to be little doubt that many different xenobiotic and plant derived organosulfur compounds have enormous benefits for in vitro biological processes and for a multitude of diseases. Even though many of these organosulfurs are quite distinct structurally, whether the multitudes of altered processes are associated with the common sulfur-moiety remains to be defined. The importance of understanding alterations of different processes by organosulfurs is based on the increasing number of reports over the past decade on alteration of diseases by xenobiotic, food, and complex organosulfur chemicals administrated directly to animals. Since the enormity of processes and diseases reportedly altered by 2-ME simply precludes a coherent single review, it was concluded that to be informative, different areas should be reviewed separately, with the goal of a final review in which to attempt to encompass all findings. To this end, preventive and therapeutic values of 2-ME were recently compared to other xenobiotic organosulfurs for cancers induced by different etiologic agents (Click, 2013). To further encompass other subject-areas, the present review will focus on in vitro alterations of immune functions of species other than mice.

2. Results and discussion

Species other than murine in which 2-ME was found to alter in vitro immune functions – fish, amphibian, reptile, avian, whales, dolphins, rat, hamster, rabbit, guinea pigs, feline, canine, porcine, ovine, bovine, and human – are summarized in Table 1; a discussion of each follows.

2.1. Fish

In general, 2-ME was not routinely part of culture media in which immune functions of different fish were studied. 50 μM in medium containing a mixture of fetal bovine serum (FBS) + autologous serum was found to optimize in vitro responses of kidney leucocytes of rainbow trout and for propagating and subculturing fibroblast-like cells from trout fins (Estepa and Coll, 1992). The fin cells were of value for culturing viral hemorrhagic septicemia virus (VHSV) and for

Table 1
2-ME doses for different animal species.

Animal	2-ME dose[a]	Purpose for use
Fish	50 μM	Immune functions enhanced, evolution, cell propagation
Amphibian	50 μM, 20 mM	Immune functions enhanced, ontogenesis, evolution
Reptiles	0, 5 μM, 70 mM	Immune functions enhanced, phylogeny, evolution
Avian	0, 10 μM, 50 μM	Immune functions enhanced, evolution, calcium signaling
Whales/dolphins	0, 7 mM	Immune functions enhanced, chemical toxins
Mice	ca. 50 μM	Immune functions enhanced, multitude of other processes
Rat	ca. 50 μM	Immune functions enhanced
Chinese hamster Rabbit Guinea pig	ca. 50 μM	Immune functions enhanced cell propagation
Feline	ca. 50 μM	Immune functions enhanced
Canine	0	None
Swine	ca. 50 μM	Immune functions enhanced, cell propagation
Ovine	ca. 50 μM	Immune functions enhanced
Bovine	ca. 50 μM	Immune functions enhanced
Human	0, 50 μM	Immune functions enhanced, AIDS, cell propagation

[a] Concentration of 2ME in culture media used for in vitro tests.

potential use as VHSV-antigen presenting cells (Estepa et al., 1993). In a model for human precursor-B (pre-B), acute lymphoblastic leukemia (ALL), also in 50 μM 2-ME plus a mixture of FBS and autologous sera, pre-B, ALL progenitor cells of transgenic zebrafish were generated and expanded as leukemic colonies in agar (Sabaawy et al., 2006).

2.2. Amphibian

Urodele amphibians have weak and sluggish immune responses compared to mammals and anuran amphibians. Using a new culture medium (Salvadori and Tournefier, 1996) supplemented with 20 mM 2-ME in place of Leibovitz L-15 medium, lymphocytes of a salamander, the Mexican axolotl (*Ambystoma mexicanum*) were stimulated to proliferate in vitro by the lymphoid mitogens, lipopolysaccharide (LPS), phytohaemagglutinin (PHA), concanavalin A (Con A), and two superantigens, staphylococcal enterotoxins A and B (SEA and SEB). LPS induced B-cells to proliferate and produce antibodies similarly throughout ontogenesis. In contrast, response to PHA and Con A by spleen cells from young axolotls was absent for the first 10 months, suggesting that maturation and/or migration processes underlie T-cell ontogenesis. Activation of the primitive, axolotl lymphocytes by SEA and SEB suggests a conservation of molecular structures in superantigen presentation and recognition. Splenocytes and thymocytes from a different species, *Xenopus laevis*, proliferated when stimulated with PHA and Con A in Leibovitz L-15 culture medium supplemented with 50 μM 2-ME (Green and Cohen, 1979). Proliferation of splenocytes at different developmental stages indicated that in contrast to those in mice, reactivity paralleled organ lymphopoiesis. A similar pattern was found for the weak responses of larval thymocytes (Rollins-Smith et al., 1984).

2.3. Reptiles

Spleen cells from the adult lizard (*Chalcides ocellatus*) produced antibodies to rat RBC in a primary in vitro response. Importantly, the 2-ME enhanced response occurred in 10% autologous sera and paralleled that in situ (Saad and El Ridi, 1988). Other research with lizards found that mixed lymphocyte culture (MLC) responses in media supplemented with 50 μM 2-ME defined a phylogenetic tree fully consistent with the phylogenetic relationships of Podarcis as determined by parallel analyses of mitochondrial DNA sequences (Valakos et al., 2007). Likewise, snakes were found to possess strong MLC activating determinants in the presence of 50 μM 2-ME and autologous serum, which were found to not be as diverse or as mutually and unilaterally stimulatory as those in higher mammals (Farag and El Ridi, 1985). In contrast, PBL from alligators underwent mitogenesis when cultured in vitro with Con A, PHA, LPS, pokeweed mitogen (PWM), purified protein derivative (PPD) and in two-way MLC in medium supplemented with adult alligator serum, but in the absence of 2-ME (Cuchens and Clem, 1979).

The presence of phagocytic IgM + cells in fish and amphibians suggested a developmental relationship between B cells and macrophages and has led to the hypothesis that B cells evolved from a phagocytic predecessor. This raised the question of when, evolutionarily, was phagocytic capacity lost by B cells. This was evaluated with red-eared slider

(turtle) B cells. The results indicated that ectothermic vertebrates use phagocytic B cells as part of a robust innate immune response when cultured in 70 mM 2-ME (Zimmerman et al., 2010).

2.4. Avian

Early MLC studies designed to characterize the major histocompatibility complex (MHC) of chickens were done in RPMI 1640 medium that was not supplemented with serum or with any sulfhydryl agent. The conclusions derived from the strain combinations used were that all stimulatory antigens were MHC-linked (Miggiano et al., 1974). Under the same one-way MLC culture conditions, the age of the donor of stimulator cells was important in specific combinations (cells from young chickens were either minimally or non-stimulatory). In addition, the age of responder cells was also crucial for specific combinations; i.e., was dependent upon the genetics of the responder strain (Bacon and Lee, 1981). In contrast to the inability to detect non-MHC antigens, use of an enriched culture medium (Modified Dulbecco's supplemented with human serum albumin (HSA) and the 2-ME equivalent sulfhydryl, alpha-thioglycerol (Goodman and Weigle, 1977), led to the definition of MLC stimulatory antigens controlled by loci not linked to the MHC (Schou, 1980). This is in agreement with the dependence on 'richness of media' for the stimulation of murine spleen cells by multiple non-MHC linked loci; namely, *mixed lymphocyte stimulatory* (*Mls*) (Festenstein, 1973) loci (Click et al., 1982) and *theta-1* locus (*Thy-1*) (Peck and Click, 1973b) that encode MMTV and theta stimulatory antigens, respectively. Alteration of Schou's culture system (1980) by replacing HSA and alpha-thioglycerol with 2% chicken serum and 2-ME resulted in mitogen stimulation that was equivalently detected by bromodeoxyuridine (BrdU) incorporation/flow cytometry and 3H-thymidine incorporation (Motobu et al., 2002). The BrdU assay allows measurement of proliferation as well as identification of cell surface marker antigens on the proliferating cells.

Calcium signaling pathways activated differentially by different agonists in the chicken DT40 pre-B-cell line were found to depend on growth conditions. Addition of 1% chicken serum to the medium increased amplitudes of calcium responses and enhanced the sustained phase to agonstic αIgM. Addition of 2-ME (final concentration of 10 μM) also resulted in large increases in the amplitude responses to αIgM, but only transient responses to agonstic H_2O_2, and did not affect responses to a third agonstic, thapsigargin (Kubista et al., 1998).

2.5. Whales/dolphins

Proliferation of lymphocytes from peripheral blood, spleen, and thymus of beluga whales (*Delphinapterus leucas*) induced by PHA, Con A and LPS in FBS was not enhanced by 2-ME. Stimulation by PWM, however, was enhanced. In addition, proliferation in medium supplemented with autologous serum in place of FBS was suppressed (DeGuise et al., 1996). In contrast, the in vitro depressed, lymphoid responses to Con A and PHA of free-ranging dolphins exposed to chemical contaminants, PCBs and DDT, were defined in medium supplemented to contain 7 mM 2-ME (Lahvis et al., 1995).

There were no reasons given for why a high concentration of 2-ME was used.

2.6. Rats

Alteration of proliferation of rat lymphoid cells by 2-ME appears to depend upon culture conditions. In one report proliferation of cells stimulated by mitogens in FBS was not enhanced by 2-ME (Liu et al., 1996); these results are just the opposite those reported by many others (Aidoo et al., 1989, 1991; Franklin et al., 1990; Kraus et al., 1985; Middleton and Bullock, 1984). Separated plastic-adherent spleen cells from suckling rats suppressed the mitogenic response of adult cells to Con A; a suppression that was abolished by the addition of 2-ME to the cultures (Middleton and Bullock, 1984). The presence of 2-ME also decreased the frequency of sister chromatin exchange in PHA stimulated cells (Aidoo et al., 1989, 1991) and partially prevented loss of Con A induced proliferation due to toxic effects of hyperoxia of different normobaric oxygen concentrations (Kraus et al., 1985). This reversal by 2-ME was similar to that found for cells of mice exposed to normobaric oxygen (Gougerot-Pocidalo et al., 1985) or mice exposed to 20 ppm nitrogen dioxide (Azoulay-Dupuis et al., 1987). Interestingly, the murine depressed response caused by normobaric oxygen was also reversed by L-cysteine and rGSH, whereas that due to nitrogen dioxide was reversed by L-cysteine and not by rGSH.

Rat MLC stimulation was absolutely dependent on 2-ME in serum-free medium and it enhanced MLC in FBS (Lindsay and Allardyce, 1979; Soulillou et al., 1975) or in 5% fresh, homologous serum (Holt et al., 1981; Lindsay and Allardyce, 1979; Mason et al., 1981). Further, delayed addition also enhanced MLC proliferation (Soulillou et al., 1975), suggesting that it has effects after Ag recognition similar to that found with human cells (Messina and Lawrence, 1992; Smyth, 1991). It had no effect on the function of cytotoxic T cells in cytotoxic medicated lympholysis (CML) (Soulillou et al., 1975). Phenotypic and functional dendritic cells identified in rat lymph node (Klinkert et al., 1980) and rat thoracic duct lymph (Mason et al., 1981) were found to be the stimulator cells for MLC, just as they were for mouse spleens (Steinman and Witmer, 1978). The murine MLC was done in medium supplemented with mouse serum + 2-ME, whereas MLCs of the rat were done in horse serum in the absence of 2-ME (lymph node) or in rat serum + 2-ME (thoracic lymph). Generation of natural killer (NK) cells from precursors in long-term rat bone marrow cultures (Van den Brink et al., 1990) or lymphokine-activated killer (LAK) cells in splenocyte cultures induced by interleukin-2 (IL-2) (Kuppen et al., 1991) was achieved in the presence of 2-ME; the latter induction was absolutely dependent upon 2-ME. In addition, differences in levels of LAK responses of five different rat strains indicated that detection of a genetic component also depended upon the presence of 2-ME (Kuppen et al., 1991).

2.7. Chinese hamster, rabbit, guinea pigs

Addition of 2-ME to a final concentration of 40 μM to Chinese hamster lymphocyte cultures resulted in a remarkable increase in lymphocyte proliferation. A similar increase was obtained at a final concentration of about 4 mM cysteine (De Jong and Van der Meer, 1984), with the results confirming those originally found with murine MLC (Heber-Katz and Click, 1972; Peck et al., 1973).

As with hamsters, there are few reports on 2-Me alteration of rabbit lymphocyte functions in vitro. Characterization of organs possessing cells with good responder and stimulator MLC functions was done in rabbit serum without added 2-ME (Milthorp and Richter, 1979). Further investigations found that 2-ME enhanced: (a) PHA/Con A mitogen responses in serum free (Dasch and Stavitsky, 1983), FBS (Liu et al., 1996; McNicholas et al., 1981), or autologous-serum supplemented medium (McNicholas et al., 1981); (b) secondary antibody responses in rabbit serum supplemented medium (McNicholas et al., 1981); (c) MLC responses in FBS supplemented medium (McNicholas et al., 1981); and (d) anti-immunoglobulin (anti-Ig) activated responses in a serum-free medium (Dasch and Stavitsky, 1983). With a more enriched medium, Iscove's Modified Dulbecco's Medium supplemented with albumin, transferrin, insulin, zinc, 2-ME, and 0.1% FBS, proliferation induced by PHA was 3 to 10 times better than that found in FBS-supplemented RPMI 1640. Under these conditions, proliferation induced by PHA did not appear to require the presence of accessory cells (Stiekema and Kapsenberg, 1987). A direct assessment of dendritic and peritoneal macrophage cells on PHA/Con A induced responses found that macrophages suppressed proliferation; a suppression that was eliminated by adding both 2-ME and indomethacin to the cultures (Kapsenberg et al., 1985).

The ability of guinea-pig spleen and lymph node cells to undergo a proliferative response in vitro induced by Con A, LPS, specific antigens, and in MLC was significantly increased by 2-ME in either serum free (Gregerson et al., 1975), FBS (Burger and Shevach, 1980; Gregerson et al., 1975; Schäfer and Burger, 1991) or autologous serum (Burger and Shevach, 1980) supplemented media. Further, alloreactive CD4 T-cell lines were generated in MLC in medium supplemented with 2-ME and FBS (Schäfer and Burger, 1991). 2-ME also enhanced PPD-induced proliferation (Hasløv et al., 1983) and was mitogenic (Gregerson et al., 1975; Hasløv et al., 1983) for lymphocytes, a finding that supports the findings (Goodman and Weigle, 1977) reported for murine spleen cells cultured in RPMI that were fed daily with a nutritional cocktail. Although these murine results conflict with those obtained by ourselves and by other groups using culture media (a) considered superior to RPMI, (b) not disrupted by daily feeding, and (c) not supplemented with heterologous serum (Heber-Katz and Click, 1972; Katz-Heber et al., 1973; Peck and Click, 1973a; Peck et al., 1973), there may be genetic control(s) that determine which strains possess autoactivity (Click, unpublished). Indeed, mitogenic effects on lymph node cells cultured in FBS, but not in guinea pig serum, in the absence of specific antigenic stimuli resulted in the development of NK cells that was enhanced by 2-ME. Generation of cytotoxic cells in autologous serum occurred only when responding cells were cultured in the presence of stimulatory antigens (Altman and Rapp, 1978).

2.8. Feline, canine

Culture conditions were optimized to assess cell-mediated immune responses of feline lymphocytes after vaccination with attenuated Nobivac vaccine, Tricat. Characterization of

lymphoproliferative responses of positive and negative CD8 cells to inactivated or to infectious feline panleukopenia virus, feline calicivirus, and feline herpesvirus-1 antigens was undertaken in medium supplemented with FBS and 2-ME. Responses of different CD8 positive and negative cells were found to vary both with the strain and status of virus—live/attenuated (Vermeulen et al., 2012).

A comparison of canine lymphocytic responses induced by mitogens, antigens or in MLC has apparently never been assessed in the presence of 2-ME or any other sulfhydryl compound. This is the one species in which essentially all immune functions in vitro were and are still done in media not supplemented with sulfhydryl agents.

2.9. Swine

Medium supplemented with 2-ME was used for studies on age-related changes in mitogen-induced lymphocyte proliferation (Hoskinson et al., 1990) as well as to discriminate different subsets of cytolytic cells in naive and in pseudorabies virus (PRV) sensitized pigs (de Bruin et al., 2000). Both cytolysis by NK cells and cytolysis by classical PRV-specific CTL were detected in PRV-sensitized pigs, whereas cytolysis by cells of naive pigs was primarily due to NK cells induced by IL-2 stimulation of PBL (de Bruin et al., 2000). In addition, two lymphoblastoid cell lines isolated from pigs were found to depend upon the presence of 2-ME for cell division and maintenance in culture for over 20 months. These cell lines were characterized by their cell surface antigens, ability to stimulate in MLC, and production of immunoglobulin (Hammerberg et al., 1985).

2.10. Sheep

Unlike other species, PBL responses of a given sheep to PHA or Con A were stated "to unpredictably vary considerably". Indeed in a longitudinal series, Con A responses in many cases were sporadically depressed. Addition of adherent cells was able to fully restore the depressed responses; addition of 2-ME was able to partially restore them (Fiscus et al., 1982). As a means to overcome some of these variations, optimization of culture conditions was undertaken by starting with culture medium that was considerably richer than the commonly used RPMI 1640. PHA stimulated proliferation in tissue culture medium 199 (TCM-199) supplemented with sodium pyruvate, horse serum, and 2-ME was 2.5-fold greater than that in RPMI supplemented with FBS, whereas the response to Con A was only equal to or slightly better (Gottshall and Hansen, 1994). Part of this enhanced reactivity may have resulted from 2-ME benefits for dendritic cells. Their generation from sheep PBL was like that of other species, done in the presence of 2-ME (Chan et al., 2002).

2.11. Bovine

In vitro proliferation of sensitized lymphocytes isolated from cows vaccinated with anaplasma antigens (Buening, 1977) and antibody synthesis specific for bovine coronavirus antigens (Larsson et al., 1992) was enhanced by 50 μM 2-ME. Further, 2-ME enhanced in vitro IgM synthesis by bovine lymphocytes stimulated with PWM (Larsson et al., 1992;

Stabel et al., 1991), whereas no enhancement occurred when reduced glutathione was added (Stabel et al., 1991). Spontaneous proliferation of PBL in 4 of 7 animals was induced by 2-Me (Larsson et al., 1992), whereas proliferation to bovine diarrhea viral antigens was achieved in cultures without 2-ME or in cultures with 0.5 or 5 μM, but not 50 μM (Larsson et al., 1992).

For a different set of questions relative for mastitis in dairy cattle, PBL exposed to a relatively low dose (5 ng/ml) of staphylococcal enterotoxin C1 (SEC1) in medium containing 50 μM 2-ME resulted not only in proliferation but by day 10, Tregs were induced (Seo et al., 2007) and monocytes were converted into plastic-adherent dendritic cells (Seo et al., 2009). These results have relevance for potential mechanisms by which staphylococcal infections of mammary glands may contribute to evasion of the immune response and affect the outcome of infection.

2.12. Human

2-ME added at the initiation of serum-supplemented MLC or mitogen-stimulated human PBL cultures resulted in the inhibition of proliferation or only a small enhancement, if any (Axelsson et al., 1976; Liu et al., 1996; Messina and Lawrence, 1992). However, if (a) added to MLC 2–3 days after initiation (Axelsson et al., 1976) or (b) added to Con A or PHA stimulated PBL that was cultured continuously in the presence of buthionine sulfoximine (BSO) or in media with suboptimal doses of cystine (Messina and Lawrence, 1992), a marked enhancement resulted. These results plus those made with rat MLC (Soulillou et al., 1975) led to the suggestion that a critical reducing equivalent of thiols is necessary for DNA synthesis but not lymphocyte activation (Messina and Lawrence, 1992; Smyth, 1991). In contrast, under essentially identical culture conditions of human PBL (RPMI + FBS), when 2-ME was added at the initiation of culture, it enhanced proliferation induced by exogenous IL-2 or OKT-3 mAB (Smyth, 1991). Further, 2-ME was found to be an essential ingredient in the serum-free culture media that supported long-term proliferation of human, gibbon, and marmoset T and B cell lines (Brown et al., 1983). Likewise, 2-ME supported the secondary antibody response of PBL stimulated with influenza in serum-free culture medium (Weiler et al., 1985).

In a different set of investigations, 2-ME prevented the suppressive effect of CD8^{+} T-cells (induced by treating PBL with IFNα) on PWM induced polyclonal immunoglobulin production and on proliferation of T cells induced in MLC or by mitogens (Schnaper et al., 1983). This inhibition is similar to that obtained with a 2-ME sensitive, soluble factor produced by Con A stimulated cells from normal individuals (Warrington et al., 1983) as well as by cultured macrophages of AIDS/ARC patients after interaction with suppressor CD8^{+} T-cells (Laurence et al., 1983). The inhibition by AIDS/ARC plasma and suppressor cells has been postulated to contribute to the decline of T cells as reflected by low levels of T cell precursor expansion as colonies in agar (Wu et al., 1989). Addition of 2-ME and to a lesser extent, N-acetylcysteine (NAc) to cultures enhanced the number and growth of T-cell precursors—2-ME caused a 400% increase and NAc a 200% increase. A similar 2-ME enhancement of T-cell colony formation in a semi-solid agar matrix was found for normal

human PBL stimulated with various mitogens (Tice and Davey, 1983). These results coupled with the beneficial outcome of AIDS patients treated with NAc (Herzenberg et al., 2005) raise the question: What would the value of a 2-ME treatment be for AIDS/ARC!

3. Conclusions

Addition of 2-ME to culture media allowed fruitful investigations in different animal species and was in some cases essential for in vitro investigation of a process; not unlike that observed in murine models. In general, processes most dependent upon 2-ME were those investigated in serum free medium, followed by those in autologous serum and lastly those in FBS supplemented media. Because of the mitogenic activity of FBS as well as its absence from in situ milieu of species other than bovine, in vitro results most relevant for in situ events would be expected to be obtained in autologous serum. In addition, the sensitivity for detection of weak antigenic determinants was increased by the use of culture media that was enriched relative to the commonly used RPMI 1640. Adaptation of available enriched media along with the ability to maintain functional activity in media supplemented with homologous, fresh or stored sera (murine sera maintained growth activities when frozen in the presence of 2-ME (Peck and Click, 1973a) indicates that the use of FBS should be curtailed, especially for in vitro investigations of situ, 2-ME altered processes. With exceptions of salamander (20 mM), turtles (70 mM) and dolphins (7 mM), optimal concentrations of 2-ME for all species, including mice, were in the low μM range. For the non-murine models discussed herein, 2-Me appears to be used primarily to increase the sensitivity of culture environments as a means to enhance various processes, some unique to given species, so they can be investigated in vitro. There were very few studies with the non-murine species delegated to defining mechanisms of action—this seems to be a question exclusively investigated with murine systems. It is anticipated that the subject area of mechanisms will require an extensive analysis of multiple processes and then presented in a full length manuscript. Such analysis will require consideration of 2-ME functions directly or indirectly: (a) as a free radical inhibitor/scavenger of reactive oxygen species (this is one of the oldest hypotheses) (Herman, 1980); (b) by regulating gene expression (Sen, 2000) after conversion to simple sulfur molecules, such as hydrogen sulfide (Wallace et al., 2012) and/or sulfane sulfur (Toohey, 1989), molecules that may have relevance for the new hypothesis that Satellite DNA ('junk DNA") acts as controlling elements via miRNA (Pezer et al., 2012); and/or (c) by maintaining critical sulfhydryl-disulfide configuration of (i) nature's endogenous thiols – glutathione and thioredoxin – at functional concentrations and proper cellular redox balance (currently perhaps the most generally accepted hypothesis (see Ref. in Click, 2010); and (ii) 'allosteric disulphide bonds' of cytoplasmic enzymes (Hogg, 2013) and/or cell membrane proteins in functional states (see Ref. in Click, 2010). Importantly, any hypotheses will need to consider that in vitro, 2-ME forms "self" or mixed disulfides (with cysteine), and that those disulfides that are bioactive are structurally susceptible

to enzymatic conversion (Toohey, 1986) to hypothesized sulfane sulfur or hydrogen sulfide.

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